國立交通大學

生物科技學系

博士論文

Lactobacillus casei rhamnosus 在腸胃道疾病中之功能性角色

Functional roles of *Lactobacillus casei rhamnosus* in gastrointestinal disease

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中華民國九十八年七月

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國　立　交　通　大　學

生　物　科　技　學　系

博　士　論　文

A thesis

Submitted to Department of Biological Science and Technology

College of Biological Science and Technology

National Chiao Tung University

in partial Fulfillment of the Requirements

for the Degree of PhD

in

Biological Science and Technology

July 2009

Hsinchu, Taiwan, Republic of China

中華民國九十八年七月

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摘　要

宿主的健康狀態與其腸道菌相有極為密切的關係，以益生菌來預防及治療腸胃道感染性或免疫性疾病已行之有年且已被許多研究證實其效果。不同商品化的益生菌對感染性疾病的預防有不同程度的效益，但以益生菌增補來達到預防腸胃道疾病的目標是可以確定的。益生菌是如何達到幫助宿主減少罹患腸胃道感染性或免疫性疾病之機制至今仍不清楚。可能是經由刺激宿主特異性與非特異性的免疫能力；或是益生菌自己合成一些抗菌物質，與病原菌競爭營養源，甚或抑制病原菌附著及侵入宿主之黏膜上皮組織等方式來達成。本實驗之目的在於探討不同 *Lactobacillus* strains 在宿主腸胃道疾病預防或治療上所扮演之功能性角色。首先，藉由比較長期增補三種不同市售益生菌產品對宿主感染性疾病影響之研究顯示：長期增補 *L. rhamnosus* T cell-1 對於預防病毒性感染有顯著成效；以混合多種/株乳酸菌之益生菌製品，則對小兒腸胃道疾病有極佳之預防效果。*L.*

casei rhamnosus 不論對小兒病毒性、細菌性、腸胃道感染或呼吸道感染性疾病的預防效果都最為顯著。我們以臨床成效最佳之 *L. casei rhamnosus* 為主要研究菌種，探討以之對抗宿主病原細菌之可能機制。將 *L. casei rhamnosus* 分別與 *E. Coli* ATCC25922、*Bacteroides fragilis* 及 *C. difficile* 等不同腸道致病菌死菌共培養，結果發現在不同腸道致病菌存在下，*L. casei rhamnosus* 抗菌能力並不受腸道致病菌誘導而發生改變，顯示 *L. casei rhamnosus* 在不需病原菌共同存在下即可分泌抗菌物質達成抗菌之效益。最後，我們亦證明 *L. casei rhamnosus* 預防或治療腸胃道發炎性疾病之可能性。*L. casei rhamnosus* 可分泌具熱穩定性之促細胞凋亡因子 (*Lcr*$S_{5\text{-}30}$)，分子量介於 5-30 kDa。此胞外因子 *Lcr*$S_{5\text{-}30}$ 可特異性誘導淋巴球或單核球以粒線體凋亡途徑進行凋亡，卻不會誘導腸黏膜上皮細胞發生凋亡。以脂多醣刺激淋巴球或單核球，在 *Lcr*$S_{5\text{-}30}$ 存在下可有效抑制發炎細胞激素的產生，有趣的是 *Lcr*$S_{5\text{-}30}$ 亦可同時誘導抗發炎細胞激素 β1 轉化生長因子之分泌，而此 β1 轉化生長因子之分泌並非誘導免疫細胞凋亡的主因，顯示細胞凋亡的效應乃由 *Lcr*$S_{5\text{-}30}$ 所促成。

Functional roles of *Lactobaciilus casei rhamnosus* in gastrointestinal disease

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ABSTRACT

Some strains of *Lactobacilli* have been widely used as probiotics expecting to improve certain immune functions in humans and animals. Probiotics are microorganisms which have benefits on the health of the host beyond inherent general nutrition. The effects of probiotics can be direct or indirect through modulation of the endogenous flora or of the immune system. Therefore, the purposes of this study are to understand how *Lactobacillus* strains alter enteric bacterial flor aand the immunomodulating capacity, and improve gastrointestinal infectious or immune diseases. First, the investigation showed that long-term consumption of *L. rhamnosus* T cell-1 decreased the incidence of bacterial infection; a multi-species probiotic reduced gastrointestinal disease significantly; and *L. casei rhamnosus* can widespread control including bacterial, viral and respiratory infections. Therefore, *L. casei rhamnosus* has been intensively studied and thought to be a useful species in clinical and nutritional fields. To determine the antimicrobial activity of *Lactobacillus* strains when coclutured with various

ratio of three kind of pathogens. The results indicated that *Lcr* promoted the greatest antimicrobial activities and there were no significant differences on the size of the inhibition halos in *Lcr* cocultured with different pathogens or *Lcr* cultured alone. Thus, pathogens cannot stimulate the antimicrobial capacities of the *Lcr* strains. The antimicrobial capacities of *Lcr* are non pathogenesis-inducible. Finally, probiotic *L. casei rhamnosus* produced heat-stable molecules (MW range 5-30 kDa) that promoted immune cell apoptosis without affecting intestinal epithelial cells. *Lcr*$S_{5\text{-}30}$ triggered apoptosis by a mitochondrial pathway, but not via TGF-β signaling pathway. *Lcr*$S_{5\text{-}30}$ also inhibited LPS-induced inflammatory cytokines by immune cells. Thus, *Lcr*$S_{5\text{-}30}$ promotes apoptosis of immune cells, and suggests probiotics-based regimens for prevention of inflammatory bowel disease.

致　　謝

真的是在博士班的這幾年世界變化太大，一路跌跌撞撞；在即將畢業的此刻，心裡突然害怕了起來！就像一直吵著要離開父母，企求獨立的小孩，當真的放手讓你去闖的時候，心裡的忐忑又讓自己想再次躲進保護當中。學海無涯啊！但，這次，是真的真的是要挺起背膀，鼓起勇氣去承擔，去開創的時候了！

感謝廖光文老師將我帶進這個領域，多年來耐心的指導，讓我在研究上或待人處事上都獲益良多。感謝東華大學彭國証老師在我身陷迷途之中給我一臂之力的拉拔，在我最徬徨無助時給予穩定的力量，彭老師的指導和幫助讓我得以順利完成博士學位，也讓我在研究上得到許多的啟發。中山醫學大學呂鋒洲老師是我研究及心靈上的導師，老師治學和治事的態度，將是我一輩子追隨的典範！感謝袁俊傑老師、張銘一老師、慈濟林俊松醫師的指導和啟發，使我的論文和研究得以更加完整。

感謝一起打拼的同學靜宜、崇仁、玄舜學長、宜仁老師，你們的鼓勵和扶持讓我心中寬慰許多，感謝學弟妹于鈴、彥谷、家弘、朝陽、博元、惠如、家慧、玉衡、玉暄、文祥、婷方、宏勳、豐吉、怡秀、惠儂、Shibu 在生活上的協助與支持。在實驗研究的壓力鍋中，生活中總有許多的驚喜與快樂、困境與苦悶，這一路因為有您們才顯得精采而難忘。

最後，要感謝我親愛的、辛苦的父母與老公，您們的相伴與支持是我最大的動力，而如果博士學位是種榮耀，這是我與您們共享與共得的!

目　　錄

中文摘要 ……I

Abstract ……III

致謝……V

目錄 ……VI

Overview **……1**

Gut immune system and the intestinal microflora ……2

Antimicrobial effects of host and probiotics in the intestinal tract ……6

Anti-inflammatory effects of host and probiotics in the intestinal tract ……10

Reference ……16

Different effects of probiotic species / strains on infections in preschool children: a double-blind, randomized, controlled study

Abstract ……29

Introduction ……30

Methods ……33

Results ……37

Discussion ……40

Acknowledgements ……44

Figures and Legends ……45

References ……57

Innate antimicrobial activity of *L. casei rhamnosus* against *Staphylococcus aureus* and *Escherichia Coli*

Abstract ……63

Introduction ……...64

Materials and Methods ……..67

Results and Discussion ……...70

Figures and Legends ……75

References ……..82

Preferential promotion of apoptosis of monocytes by *Lactobacillus casei rhamnosus* soluble factors

Abstract ……86

Introduction ……...88

Materials and Methods ……..91

Results ……...99

Discussion ……105

Figures and Legends ……111

References ……130

Curriculum vitae ……134

OVERVIEW

Probiotics have been defined as viable microorganisms that have beneficial effects in the prevention and treatment of specific pathologic conditions (Havenaar et al., 1992). The rationale of probiotic benefits is normalizing unbalanced indigenous microflora by administration specific xeno-probiotic strains to gastrointestine. Many microorganisms have been used or considered as probiotics, they are strains of lactic acid bacteria (e.g., *Lactobacillus, Bifidobacterium and Streptococcus*) and nonpathogenic yeast, *Saccharomyces boulardii*. *S. thermophilus* and *L. bulgaricus*, generally used in the dairy food industry, were among the first to be studied. Other strains that have been commonly used are *B. bifidum, B. longum, Enterococcus faecium, S. boulardii, L. acidophilus, L. casei, L. rhamnosus* and *Lactobacillus GG*. Among them, *Lactobacillus* is the most popular genus. The probiotic preparation may contain one species/strain to various combinations of different microorganisms' strains. Both viable and biologically active microorganisms are required at the target site in the host, it is essential that the probiotic be able to withstand the host's natural barriers against ingested bacteria. Probiotics have been studied in the treatment and prevention of various allergic symptoms and of inflammatory bowel disease. The character and effectors of probiotics would be described in this dissertation.

1. Gut immune system and the intestinal microflora

Innate immune recognition and host defense within the intestinal mucosa represent a very complex issue. The presence of the dense and highly dynamic microbial flora requires mechanisms of discrimination between infection with potentially harmful microbes and commensal colonization. Inappropriate responses may lead to inflammation, tissue destruction and organ dysfunction. However, lack of innate immune recognition and the failure to mount efficient host responses may lead to overgrowth of commensal bacteria, breakage of the epithelial barrier and invasive enteropathogen infection (Kobayashi et al., 2005; Viala et al., 2004). Thus, innate immune recognition within the intestinal tract needs to be restricted to potentially dangerous microbial pathogens, and antimicrobial effectors mechanisms require a delicate balance to maintain the host-microbe homeostasis. A better understanding of the processes and factors involved in mucosal pathogen-associated molecular pattern-recognition receptors (PRR) detection may help to explain the lifelong host-microbe homeostasis in the healthy individual, but also the pathogenesis of prevalent human intestinal diseases.

1.1 The intestinal microflora influences gut immune system

The intestinal lumen is colonized by a large and very dynamic microbial flora. The

number and composition changes considerably within the gastrointestinal tract with moderate numbers but complex bacterial communities in the oral cavity, low to very low numbers in the esophagus, stomach and duodenum and increasing amounts of mostly Gram-positive bacteria along the jejunum and ileum (10^4–10^7/g content). The ileocaecal valve represents an important physical barrier to the caecum and colon which are heavily colonized by a highly diverse and mostly anaerobic Gram-negative flora reaching a density of 10^{14} bacteria/ml and exerting a strong fermenting activity. The diversity of species encompasses around 400 different bacteria (Simon and Gorbach, 1984). Within these, 30–40 different bacterial species make up for 99% of the total population of the intestine (Guarner 2006). The predominant bacteria are *Bifidobacterium* spp., *Eubacterium* spp., *Clostridium* spp., *Lactobacillus* spp., and *Bacteroides* spp., and only relatively low numbers (<1%) of the *Enterococcus* spp. or members of *Enterobacteriaceae* are found.

The development of the adaptive gut immune system is also deeply influenced by the intestinal microflora. Germfree mice show lower levels of immune cells and soluble immunoglobulins in the intestinal tissue. This situation is rapidly reversed after microbial colonization with lymphocyte expansion, reappearance of germinal centers and rise of immunoglobulin levels (Butler et al., 2000; Fagarasan et al., 2002). The microflora also

contributes to oral tolerance to food antigens. Conventional mice but not monocolonized or germ-free mice were able to efficiently obtain tolerance after oral application of the food antigen ovalbumin (Moreau and Corthier, 1988; Rask et al., 2005). Although germfree mice have helped to investigate the role of commensal bacteria in intestinal physiology, it should be noted that microbial ligands, the structures recognized by the innate immune system, can also be found in significant concentrations in the gut of germ-free mice ingested by the sterile, but not ligand-free nutrients.

1.2 Innate immune recognition and intestinal homeostasis

The innate immune system harbors a high degree of specificity to distinguish between self and the presence of microbial organisms (Barton and Medzhitov, 2000). Specific microbial structures so-called pathogen-associated molecular patterns (PAMPs) are recognized by a variety of pattern-recognition receptors (PRRs) (Janeway et al., 2002; Gordon, 2002). However, it is important to note that the recognized microbial structures of any of the discovered receptors is not restricted to pathogens but also found in many if not all commensal microorganisms. PRRs, such as mannan-binding lectin (MBL), C-reactive protein (CRP), or serum amyloid protein (SAP), may be secreted in the extracellular fluid. Other PRRs are expressed intracellularly such as members of the family of nucleotide oligomerization domain (NOD)-like receptors like for example NOD1, NOD2, and Nalp3

and also PKR, or the helicases MDA-5 and RigI. Finally, some PRRs, e.g. members of the family of Toll-like receptors (TLRs), are expressed at the cell surface or monitor endosomal compartments (Medzhitov and Janeway, 1997). Functional PRRs are present within the mucosal surface of the intestinal tract. NOD1 and NOD2 have been shown to be expressed by epithelial cells and to contribute to intestinal homeostasis and host defense (Kobayashi et al., 2005; Maeda et al., 2005).

The identification of the first IBD gene, CARD15, codes for the NOD2 protein (Hugot et al., 2001; Ogura et al., 2001). The cytoplasmatic protein NOD2 recognizes bacterial cell-wall products, in particular peptidoglycans, and influences the activation of NF-κB. In the present studies, mice expressing the homologue mutation within *Nod2* exhibit a hyperinflammatory phenotype (Maeda et al., 2005). Also, mice lacking *Tlr4* expression showed a more severe disease in the DSS model of colitis (Rakoff-Nahoum et al., 2004). Thus, excess or absent PRR activation within the intestinal mucosal tissue may significantly contribute to organ dysfunction and gastrointestinal disease. In addition, NOD2 signaling seem to interacts with the signaling of toll-like receptors (TLRs) and that this may be one way how deficient action would actually lead to more inflammation (Watanabe et al., 2004). Furthermore, NOD2 seems to be involved in regulation of the production of endogenous antibacterial peptides such as defensins and proapoptotic

signaling cascades (Wehkamp et al., 2004).

2. Antimicrobial effects of host and probiotics in the intestinal tract

2.1 Antimicrobial effects in the intestinal tract

The mucosal surface is coated by a mucus layer produced by goblet cells and epitheial cells. Two pathways exist by which mucins are secreted into the intestinal lumen (Forstner 1995). The first results in a constitutive secretion process by mucin exocytosis, also named baseline production. The other pathway comprises packaging and storage of mucins in large secretory vesicles released following stimulation by neuroendocrine mediators or inflammatory mediators. The functional importance of the mucus layer in production of the epithelial lining was demonstrated by studies showing that mucin 2-deficient mice spontaneously develop colonic mucosal inflammation and show enhanced tumorigenesis (Van der Sluis et al., 2006; Velcich et al., 2002). Also, enhanced numbers of bacteria closely attached to the mucus were described in patients with IBD indicating reduced barrier function of the mucus layer in these patients (Schultsz et al., 1999). Mucin upregulation was detected after infection with enteropathgenic bacteria such as *Salmonella, Yersinia, or Shigella* (Mantle et al., 1989; Nutten et al., 2002). Interestingly, bacteria of the intestinal microflora, *Lactobacillus plantarum*, have been

reported to increase mucin production in epithelial cells (Mack et al., 2003).

Another important barrier is provided by the various antimicrobial peptides (e.g. α-defensins, cryptdin-related sequence peptides, lysozyme and phospholipase A2) produced by crypt Paneth cells in the intestinal tract (Bevins 2006; Hornef et al., 2004). Nizet et al. (2001) and Pütsep et al. (2002) have shown that antimicrobial peptides were involved in the control of the physiological microbial flora and mucosal homeostasis. Interestingly, reduced numbers of Paneth cells and a decrease in α-defensin production were noted in Crohn disease patients (Wehkamp et al., 2005).

2.2 Probiotics' antimicrobial effects in host intestinal tract

At least 2 mechanisms of probiotic action have been identified to mediate maintenance of the gastrointestinal microbial balance: production of antibacterial substances and competitive inhibition of pathogen and toxin adherence to the intestinal epithelium. In fact, both predominance of potentially harmful bacteria and a decrease of beneficial bacterial species such as *Lactobacillus* and *Bifidobacterium* have been identified in intestinal microbiota in patients with gastrointestinal diseases (Conte et al., 2006; Mylonaki et al., 2005; Ott et al., 2004), thus suggesting that manipulation of intestinal bacteria may provide an alternative therapy for gastrointestinal diseases

prevention and treatment.

Probiotics exert direct antibacterial effects on pathogens through production of antibacterial substances (Cotter et al., 2005; Servin, 2004). Probiotic bacteria, especially strains of *Lactobacilli,* produce acetic, lactic, and propionic acid that lower the local pH leading to inhibit growth of a wide range of Gram-negative pathogenic bacteria. Studies indicate that these probiotic-derived antibacterial substances exert their effects alone or synergistically to inhibit the growth of pathogens. Some *Lactobacillus* strains inhibit the growth of *Salmonella enterica* solely by the production of lactic acid (Makras et al., 2006). However, antibacterial effects of other strains of *Lactobacilli* may be the result of a combination of lactic acid and other unknown *Lactobacillus*-derived bactericidal substances by pH-dependent mechanism (s) (Makras et al., 2006).

On the other hand, competitive inhibition of probiotics in the gastrointestinal tract decreases adhesion of both pathogens and their toxins to the intestinal epithelium. Several strains of *Lactobacilli* and *Bifidobacteria* are able to compete with pathogenic bacteria, including *Bacteroides vulgatus, Clostridium histolyticum, C. difficile*, *Enterobacter aerogenes, Listeria monocytogenes, Staphylococcus aureus* (Collado et al., 2007), *Salmonella enterica, Yersinia enterocolitica* (Candela et al., 2005), enterotoxigenic *E.*

coli (Roselli et al., 2006), and enteropathogenic *E. coli* (Sherman et al., 2005) for intestinal epithelial cell binding, and they can displace pathogenic bacteria even if the pathogens have attached to intestinal epithelial cells prior to probiotic treatment. However, specific probiotics or probiotic combinations should be selected based on their ability to inhibit or displace a specific pathogen. Since 1 of the mechanisms underlying pathogenic bacteria binding to intestinal epithelial cells is through the interaction between bacterial lectins and carbohydrate moieties of glycoconjugate receptor molecules on the cell surface, studies have addressed whether probiotics can block pathogen binding to these receptors. Studies regarding proteinase treatment and carbohydrate competition have confirmed that probiotic binding to intestinal epithelial cells is mediated by lectin-like adhesion and proteinaceous cell surface components (Mukai et al., 2004; Sun et al., 2007; Tallon et al., 2007). For example, mannose and Galβ1-3GalNAc-specific adhesions are required for binding of *Lactobacilli* and *Bifibobacteria* to attach to intestinal epithelial cells and mucus. Thus, probiotic inhibition of pathogen adherence to intestinal cells is mediated in part by competition for lectin binding sites on glycoconjugate receptors on the cell surface. Blockade of bacterial enterotoxin binding has also been demonstrated as a mechanism with therapeutic potential. The virulence factor of enterotoxigenic *E. coli* strains is a heatlabile enterotoxin that induces traveler's diarrhea by binding to ganglioside GM1 on the surface of intestinal epithelial cells. By

using a toxin-receptor blockade strategy, an engineered probiotic bacterium was generated to express glycosyltransferase genes from *Neisseria meningitides* or *Campylobacter jejuni* in a nonpathogenic *E. coli* strain (CWG308). The recombinant *E. coli*-produced chimeric lipopolysaccharide neutralized heat-labile enterotoxin and cholera toxin in vitro, prevented enterotoxin-induced fluid secretion in ligated rabbit ileal loops in vivo (Paton et al., 2005), and reduced mortality by virulent *V. cholerae* infection in infant mice (Focarta et al., 2006).

3. Anti-inflammatory effects of host and probiotics in the intestinal tract

3.1 Anti-inflammatory effects in gut immune system

The intestinal epithelium might indeed able to sense the presence of microbial immunostimulatory molecules but rely on life-long negative regulatory mechanisms to prevent inappropriate immune activation and tissue inflammation. In recent years a large number of negative regulators of the Toll-like receptor (TLR) signaling pathway have been described. They comprise the regulation of receptor and co-receptor expression, the presence of competitive receptor antagonists, and regulatory molecules along the downstream signaling pathway, as well as the post-transcriptional down-regulation of essential intermediates within the signaling cascades (Liew et al., 2005). Importantly,

inhibitory circuits usually affect several TLRs simultaneously due to the redundant function of many signaling molecules downstream of TLRs (Sato et al., 2000). Down-regulation of the TLR4 molecule has been noted early in response to lipopolysaccharide (LPS) stimulation (Nomura et al., 2000; Hornef et al., 2002). The finding that epithelial TLR4 expression in increased in patients suffering from IBD may indicate enhanced ligand susceptibility (Cario and Podolsky, 2000; Kalis et al., 2003). Also, transmembrane TLR antagonists have been characterized. One example is ST2 that seems to inhibit TLR signaling via interaction with the adaptor molecules MyD88 or MAL. Cytokine secretion in ST2- deficient macrophages is significantly increased and ST2-deficient mice fail to develop LPS tolerance in sepsis model (Brint et al., 2004).

3.2 Anti-inflammatory effects of normal flora

Recent studies have revealed surprising results indicating a much closer interaction between the microflora and the intestinal epithelium as previously recognized (Hooper et al., 1999). Although immunostimulatory structures recognized by the innate immune system are generally produced by both, commensal and pathogenic bacteria, the synthesis of low stimulatory hypoacylated LPS isolated from a prominent gut commensal *Bacteroides fragilis* might contribute to homeostasis (Weintraub et al., 1989). The commensal bacterium *Bacteroides thetaiotaomicron* enhances the nuclear export of the

NF-κB subunit p65/RelA by peroxisome proliferative-activated receptor (PPAR)-γ in intestinal epithelial cells (Kelly et al., 2004), limiting NF-κB activation. Indeed, co-culture experiments indicate that *B. thetaiotaomicron* attenuates pro-inflammatory responses mediated by the pathogen *Salmonella* Enteritidis. Interestingly, ulcerative colitis patients show reduced levels of PPAR-γ expression in colonic epithelial cells, a possible explanation for the enhanced inflammation seen in these patients (Dubuquoy et al., 2003). Strikingly, non-virulent *Salmonella enterica* Pullorum was noted to block IκB-α degradation and thereby exert an anti-inflammatory effect on intestinal epithelial cells (Neish et al., 2000). Similar mechanisms may underlie the effects observed using probiotic bacteria, which beneficially affect the host by improving its intestinal microbial balance (Menard and Heyman, 2005).

3.3 Probiotics applied in inflammatory disease

Inflammatory bowel disease refers to disorders of unknown cause that are characterized by chronic or recurrent intestinal inflammation. Such disorders include ulcerative colitis, Crohn disease, and pouchitis. The mechanisms responsible for initiation and perpetuation of the inflammatory process remains unknown, but the main theory is that IBD may result from abnormal host responses to some members of the intestinal flora or from a defective mucosal barrier. Treatment may be difficult and there

is a need for new treatments to decrease the occurrence of symptoms and to prevent recurrence.

Several studies showed interesting effects of probiotics on IBD in animals. Intracolonic administration of *L. reuteri* R2LC to rats with acetic acid-induced colitis significantly decreased the disease, whereas *Lactobacillus* HLC was ineffective (Fabia et al., 1993). Administration of *L. reuteri* R2LC and *L. plantarum* DSM 9843 to rats with methotrexate-induced enterocolitis was associated with low intestinal permeability, bacterial translocation, and plasma endotoxin concentrations compared with rats with enterocolitis and no treatment (Mao et al., 1996).

A few studies were also performed in patients. In an open study, a 10-day administration of *L. rhamnosus* GG to 14 children with active or inactive Crohn disease resulted in an increase in immunoglobulin A-secreting cells to β-lactoglobulin and casein, which indicates an interaction between the probiotic and the local immune system (Malin et al., 1996). The lactobacilli did not influence the disease activity, however, because the study group was too small and the study was too short to assess accurately a clinical effect. Plein and Hotz (1993) performed a pilot, double-blind, controlled study of the efficacy of *S. boulardii* on symptoms of Crohn disease were randomly assigned to

receive either *S. boulardii* or a placebo for 7 wk in addition to the standard treatment. A significant reduction in the frequency of bowel movements and in disease activity was observed in the group receiving *S. boulardii* but not in the placebo group.

The impact on the severity of lesions was also evaluated. A combination of three *Bifidobacterium* species, four *Lactobacillus* species and *S. salivarius* ssp. *Thermophilus* (VSL#3, VSL Pharmaceuticals) has been evaluated in a single-blind study for the prevention of recurrent inflammation after surgery (Gionchetti et al., 2003). The patients either received a nonabsorbable antibiotic (rifaximin) for 3 months followed by 9 months intake of the probiotic or mesalazine for 12 months. After 1 year there was a significantly lower rate of severe endoscopic recurrence in patients treated with the antibiotic and probiotic combination. Patients with active colonic Crohn disease were treated with prednisolone on a standard schedule and were also randomized to receive *E. coli* (Nissle 1917) or placebo for 1 year (Malchow 1997). Patients in the two f groups had similar rates of remission but patients treated with prednisolone and *E. coli* had fewer relapses than patients in the placebo group. The difference was not statistically significant.

The literature on the role of probiotics in the treatment of inflammatory disease is still regarded as limited and only a few probiotic strains were shown to be efficacious in

randomized placebo-controlled clinical trials. Also, the precise mechanism by which probiotic induces remission and prevents relapse remain unknown. Future studies are needed on the effect of probiotics in maintenance of remission and understanding the molecular mechanism.

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Different effects of probiotic species / strains on infections in preschool children: a double-blind, randomized, controlled study

ABSTRACT

Treatment and prevention of pediatric infectious diseases of three commercial probiotic products were evaluated by a double-blind, randomized, controlled trial. Test subjects under age 5, 1062 in total, were distributed randomly into four groups. This investigation showed that *L. casei rhamnosus* can control bacterial, viral and respiratory infections; a multi-species probiotic reduced gastrointestinal disease significantly. Long-term consumption of *L. rhamnosus* T cell-1 decreased the incidence of bacterial infection.

Keywords: probiotics; preschooler; infections.

INTRODUCTION

Infectious diseases are the most significant illnesses for children under age 5, particularly for those attending preschool (Kvaerner et al., 2000). Poor hygiene facilities present risks for respiratory and gastrointestinal tract infectious pathogens (Nafstad et al., 1999; Pickering et al., 1986). Viral pathogens, such as respiratory syncytial virus (Law et al., 2004), human metapneumovirus (Van den Hoogen et al., 2004), influenza A virus (Wolf et al., 2006), parainfluenza viruses, and rhinoviruses are considered the major viruses that can cause respiratory tract diseases in children (Wolf et al., 2006); furthermore, rotaviruses (Medici et al., 2004), adenoviruses (Van et al., 1992), and astroviruses (Basu et al., 2003) are viral pathogens that cause gastrointestinal diseases in children. Important infectious bacteria that have been implicated in day care-associated respiratory and gastrointestinal disorders are *Streptococcus pneumoniae* (Malfroot et al., 2004), *Shigella*, *Salmonella*, *Escherichia coli* (Binsztein et al., 1999), and *Aeromonas* (Binsztein et al., 1999; Soltan Dallal and Moezardalan, 2004).

Effective remedies have been intensively investigated to reduce pediatric infectious diseases which cause illness, debility, and in extreme cases, loss of life. Managerial methods (Strachan, 1989; Brady, 2005) and probiotic supplementation are currently employed to reduce the incidences of infectious disease (Rosenfeldt et al., 2002; Hatakka et al., 2001).

It has been proven that children with mild diarrhea who consumed the combination of *L. rhamnosus* and *L. reuteri* experienced a reduction in the duration of the diarrhea (Rosenfeldt et al., 2002). A study of 6- and 36-month old children afflicted with rotavirus gastroenteritis showed that *L. reuteri* significantly shortened the duration of diarrhea (Shornikova et al.,

1997). *L. rhamnosus* and *L. acidophilus* significantly attenuated the neutrophil infiltration and lipid peroxidation during *Shigella* dysenteriae 1-induced diarrhea in rats (Moorthy et al., 2007). In addition, it was shown with the use of newborn rabbits as an experimental infection model that preventive administration of *L. casei* may, due to acceleration of a specific humoral immune response, lead to enhanced resistance to acute *E. coli* infection (Ogawa et al., 2001). These studies illustrated the various effects of different *Lactobacillus* strains on gastrointestinal infections. It has been observed that several strains of probiotics have positive influences on non-specific stimulation of the host's immunity, although the molecular mechanism has not been elucidated (Trushina et al., 2006; Christensen et al., 2006; Kim et al., 2006). Many probiotics are capable of preventing respiratory infections and reducing their severity (de Vrese et al., 2006). Also, it was reported that mitigation or prevention of pediatric infectious disease occurred when children in day care centers ingested *Lactobacillus* (Hatakka et al., 2001).

The efficacy of commercial probiotics has been brought under scrutiny, with doubt remaining that all of them possess sufficient potency necessary for adequate gastrointestinal colonization. Further evidence is required to demonstrate that strain-specific probiotics can prevent various diseases. The proper selection among mono-strain, multi-strain, or multi-species probiotics is critical for efficacy in clinical trials (Timmerman et al., 2004). Previous investigations involving the function and efficacy of probiotics on the prevention of pediatric infectious diseases were focused only on evaluating a single probiotic. So, the aim of this double blind, randomized, controlled study was to compare the efficacy of three different commercial probiotics – *Lactobacillus casei rhamnosus, Lactobacillus rhamnosus* T cell-1 and a multiple probiotic – during short- and long-term intervention. The parameters examined in this investigation were the effect of different probiotics on the incidences of bacterial and

viral infectious diseases, and more specifically, gastrointestinal and respiratory infections in preschooler. We report here that various commercial probiotics have dissimilar effects on different infectious diseases. The *L. casei rhamnosus* probiotic reduced respiratory infections, but multispecies probiotic supplementation significantly reduced gastrointestinal disease.

METHODS

Study design and ethics

This was a double-blind, randomized, controlled study, with four parallel arms, and consent letters were signed by well-informed parents. This study was approved by the Committees of the Protection of Human Subjects Institutional Review Board of Tzu-Chi University and Hospital, Hualien, Taiwan.

Participants

One thousand and sixty-two children were recruited and 76 children who did not remain in the study during the follow-up period were excluded from the investigation. Among the 986 children who completed the study, 193 were in the control group, 285 were in the *L. casei rhamnosus* group, 222 were in the *L. rhamnosus* T cell-1 group, and 286 were in the multiple probiotic group (Fig 1). The characteristics of each study group are given in Table 1. No significant differences were observed in age, male/female ratio, duration of breast-feeding, smoking in the household, family income, house area, and history of allergy. This study excluded children who previously had complicated intestinal operations or immunosuppressive therapy, or those who suffered ill effects due to complex congenital heart disease, or low immune function syndromes.

Test preparations, blinding, and randomization

The intervention lasted 7 months, from October 20, 2003 to May 31, 2004. We regarded each class as a unit, and implemented a double-blind assignment of *L. casei rhamnosus*

sachets, *L. rhamnosus* T cell-1 capsules, and multiple probiotic capsules to the children. The subjects were randomly assigned to one of four groups:

1. The *L. casei rhamnosus* group: Instructions for consumption: 2 sachets (3 g) of *L. casei rhamnosus* per day, 5 days a week;
2. The *L. rhamnosus* T cell-1 group: Instructions for consumption: 3 capsules (1.14 g) of *L. rhamnosus* T cell-1 per day, 5 days a week;
3. The multiple probiotic group: Instructions for consumption: 5 capsules (5 g) of a mix of 12 beneficial bacterial strains per day, 5 days a week;
4. The control group: No probiotic supplementation; no dietary inclusion criteria.

One *L. casei rhamnosus* sachet contained 1×10^8 cfu *L. casei rhamnosus*/g (Antibiophilus® Laboratoires Lyocentre Ltd, Aurillac, France), one *L. rhamnosus* T cell-1 capsule contained 1×10^{10} cfu *L. rhamnosus* T cell-1/g (T Cell-1 Probiotics, Chang Gung Biotechnology Corp, Taipei, Taiwan), and a multiple probiotic capsule contained 12 types of beneficial bacterial strains for the large and small intestines, including 7 different species of *Lactobacilli* (Neoangelac® 12A *Lactobacilli,* Multipower Enterprise Corp, Taipei, Taiwan). One capsule of the Neoangelac 12A *Lactobacilli* series contained 3 types of *Bifidobacteria* (2.4×10^9 cfu *B. bifidum*, 2.4×10^9 cfu *B. infantis*, 2.4×10^9 cfu *B. longum*); 7 types of *Lactobacilli* (2×10^9 cfu *L. casei*, 1.2×10^9 cfu *L. salivarius*, 1.6×10^9 cfu *L. brevis*, 2×10^9 cfu *L. plantarum*, 1.2×10^9 cfu *L. acidophilus*, 8×10^8 cfu *L. helveticus*, 2×10^9 cfu *L. rhamnosus*); 1 type of *Streptococcus* (1×10^9 cfu *S. thermophilus)* and 1 type of *Enterococcus* (1×10^9 cfu *E. faecium*). Dietary restrictions were not applied during the intervention periods.

Intervention

Nine hundred eighty-six participants were observed between January 1, 2001 and May 31, 2004. The baseline period was when these children attended day care centers from January 1, 2001 to December 31, 2002. Then, the intervention period lasted 7 months from October 20, 2003 to May 31, 2004. The short-term intervention period and the long-term intervention period were defined as interventions that lasted 3 months and 7 months, respectively, each beginning from October 20, 2003. The volunteers took the probiotic products, following the instructions on the package label. The investigated parameters were incidences of all pediatric diseases, bacterial infections, viral infections, gastrointestinal infections, and respiratory infections. Incidence frequency and episodes per person per month were described as the number of infection episodes relative to the corresponding population experience, and excluded routine immunization and other scheduled visits that were not related to infections. Infectious episode information during January 1, 2001 to May 31, 2004 was collected from the Bureau of National Health Insurance, Taiwan.

Assessment of infectious disease

Average incidence densities of 167 types of diseases were estimated for each probiotic group. The pediatric diseases were defined as gastrointestinal disease, respiratory disease, atopic disease, and dermatologic disease. Bacterial infections were lymphadenitis, acute otitis media, pneumonia, sinusitis, urinary tract infection, meningitis, and bacterial gastroenteritis, etc. Viral infections were influenza, acute pharyngotonsillitis, acute laryngitis, croup, enterovirus infection, acute bronchitis, acute bronchiolitis, encephalitis, and viral gastroenteritis, etc. Respiratory infection included 28 categories such as the common cold, acute upper respiratory infections, acute bronchitis, acute bronchiolitis, acute sinusitis, acute pharyngitis, acute tonsillitis, acute laryngitis, acute epiglottitis and influenza, et al.; gastrointestinal infections included viruses and bacteria associated with diarrhea and vomiting;

abdominal pain included 23 categories and non-infectious gastrointestinal disease; constipation included 22 categories, for a total of 50 classes.

Statistical analysis

Descriptive statistics, including the mean and standard error of the mean (S.E.M.), were determined for each of the four groups. Analysis of variance (ANOVA) was applied to all pediatric diseases, bacterial infections, viral infections, gastrointestinal infections, and respiratory infections that existed among the probiotic-treated groups and the control group during the same period and among the different periods in the same group. If a significant difference was present, the least significant difference (LSD) multiple comparison tests were used to identify specific significant groups. All statistical analyses were performed by using The Statistical Software Package for the Social Sciences, version 12.0.1 for Windows (SPSS Inc., Chicago, IL). A *P* value of < 0.05 was considered statistically significant for all analyses.

RESULTS

Effect of probiotic treatment on incidence of all pediatric disease

Preschoolers' incidences of all pediatric diseases increased after entering the day care center in every group compared with the baseline period (P>0.05, Figure 2). Preschoolers who received probiotic treatment experienced a decline in physician visits, although this was statistically insignificant between the groups during the intervention period (P>0.05, Figure 2).

Effect of probiotics on bacterial infectious disease

The incidence of bacterial infectious disease in preschoolers of every group increased significantly after entering the day care center compared to the baseline period (P<0.05, Figure 3). Preschoolers who received a single strain probiotic during the long-term intervention experienced a significant improvement in recovery from any bacterial infectious disease. The incidence of bacterial infection diagnosed by doctors in the *L. casei rhamnosus* group was fewer (mean difference, -0.15 times/month; CI_{95}, -0.27 to -0.03; P=0.021, Figure 3) as compared to the control group. The analysis of the *L. rhamnosus* T cell-1 group showed a significant decrease in those with a doctor-diagnosed bacterial infection as compared to the control group (-0.16; CI_{95}, -0.30 to -0.03; P=0.020, Figure 3). No significant differences in the incidence of doctor-diagnosed bacterial infection were observed in the multiple probiotic and control groups (P>0.05, Figure 3).

Effect of probiotics treatment on viral infectious disease

Incidences of viral infectious diseases in preschoolers increased after entering the day care center in every group as compared to the baseline period (P<0.05, Figure 4). The preschoolers receiving the *L. casei rhamnosus* treatment had 0.30 times lower odds of doctor-diagnosed viral infection than the control group during short-term intervention (-0.16; CI_{95}, -0.54 to -0.06; P=0.015, Figure 4). No significant difference was observed in the *L. rhamnosus* T cell-1 and the multiple probiotic groups (P>0.05, Figure 4).

Effect of probiotic treatment on gastrointestinal disease

There was no difference in the incidence of gastrointestinal infectious disease after preschoolers entered the day care center in the control group compared with the baseline period (P<0.05, Figure 5). However, preschoolers in the multiple probiotic group experienced a significant reduction in gastrointestinal infection both during the short-term (-0.045; CI_{95}, -0.040 to -0.040; P=0.007) and the long-term (-0.049; CI_{95}, -0.037 to -0.061; P=0.004) intervention. Single strain probiotic (*L. casei rhamnosus* and *L. rhamnosus* T cell-1, respectively) administration showed an insignificant ability to prevent disease when compared to the control group (P>0.05, Figure 5). However, the mean incidence of gastrointestinal disease significantly decreased in the group that participated in the short-term consumption of *L. casei rhamnosus* as compared to the group that consumed *L. casei rhamnosus* before entering preschool (-0.034; CI_{95}, -0.041 to -0.026; P=0.031 and -0.040; CI_{95}, -0.046 to -0.034; P=0.011, Figure 5).

Effect of probiotics on respiratory disease

The incidence of respiratory infectious disease in preschoolers increased after entering the day care center in every group as compared to the baseline period (P<0.05, Figure 6).

There was a significant difference in doctor-diagnosed respiratory infection between the *L. casei rhamnosus* and the control group, and there was also a significant reduction in both the short-term(-0.352; CI_{95}, -0.243 to -0.460; P<0.000) and the long-term(-0.309; CI_{95}, -0.200 to -0.418; P<0.000) intervention groups (Figure 6).

DISCUSSION

Many studies have highlighted the benefits of probiotic bacteria for infectious disease prevention. However, the efficacy of many commercial probiotics is suspect, due to insufficient growth of various strains in the human intestinal tract, and hardly any probiotic manufacturers have solid evidence to match their claims. But *Lactobacillus spp.* have earned the most attention and have been investigated intensively, as this bacterial genus is among the few that confer many positive rewards to the test subjects. Three *Lactobacillus spp.*, *L. casei rhamnosus*, *L. rhamnosus* T cell-1, and the Neoangelac 12A *lactobacilli* multipower are major components of probiotic formulae and are very popular in the probiotic market of Taiwan. In the interest of the good health of preschoolers and the welfare of society, the pediatric benefits of these probiotics were further investigated in this study.

The incidence of all pediatric disease increased after entering day care centers in every group. These observations support the belief that attendance at day care centers increases the risk of infections (Kvaerner et al., 2000; Nafstad et al., 1999). An interesting result was that probiotic supplementation tended to diminish the number of physician visits, especially in reducing the number of children who had a high incidence of physician visits, and concomitantly increased the number of children who had no physician visits during the intervention period (data not shown).

Gastrointestinal infections in preschool can be attributed to different factors, such as the transmission of enteropathogens by fomites, or ingestion of contaminated food or drink. Diligent hygiene was practiced by the preschoolers' families, and at all the day care centers. Furthermore, the gastrointestinal disease analysis suggested that preschool attendance did not

lead to an increased risk of infections. Meanwhile, this study clearly showed the effectiveness of the multiple probiotics in preventing gastrointestinal disease in preschoolers. Reductions of 42% and 44% were found in gastrointestinal disease in the short- and long-term intervention periods, respectively, of the multiple probiotics group. In the *L. casei rhamnosus* group, there was a decreased frequency of gastrointestinal disease in preschoolers, although statistically insignificant when compared to the control group. The children who received the single strain *L. rhamnosus* T cell-1 supplementation did not exhibit any statistically significant difference as compared to the control group.

The variety of commensal bacteria is essential to the development of gut mucosal immunity (Strachan, 1989; Noverr and Huffnagle, 2005). Previous research has shown that a combination of probiotic bacteria can stimulate the mucosal immune system, with similar conclusions being made from animal studies, and mixtures of gut microbial species can more efficiently stimulate the immune system than a single strain (Lanning et al., 2000; Kelly et al., 2005). Other reports that are in agreement with our results are the findings that the combination of *Lactobacillus rhamnosus* and *L. acidophilus* offered better protection compared to a single strain of *Lactobacilli* during a *Shigella* infection (Shornikova et al., 1997); and probiotic products containing one strain of *Lactobacillus* had less positive effects on gastrointestinal diseases because of a decreased ability to successfully colonize the gastrointestinal tract (Famularo et al., 1999).

This investigation clearly showed that single strain probiotic supplementation significantly reduced the incidence of bacterial infections by an average of 1.8 times for *L. casei rhamnosus*, and 1.92 times for *L. rhamnosus* T cell-1 during the experimental period. Some of the mechanisms that probiotics use to promote health include the synthesis of

anti-microbial substances (Sillva et al., 1987), reduction of the nutrients available for bacterial pathogens (Wilson and Perini, 1988), inhibition of adhesion and invasion of pathogens (Bernet et al., 1994), modification of toxin receptors (Pothoulakis et al., 1993), and stimulation of immune responses (Trushina et al., 2006; Christensen et al., 2006; Kim et al., 2006; Kaila et al., 1992). However, the multiple probiotic supplement had no significant effect on preventing bacterial infections. This might be attributed to antagonism among the different strains of probiotics in the multi-strain supplement (Timmerman et al., 2004).

Consumption of *L. casei rhamnosus* reduced viral infectious disease by 18% in the short-term intervention group. In the multiple probiotic and the *L. rhamnosus* T cell-1 groups, the effects of the probiotics were not strong enough to prevent viral infections. Much research has been done to study the effect of probiotics on bacterial or gastrointestinal infectious diseases, but only a few studies have examined the effect of probiotics on viral or systemic infectious diseases. de Vrese et al. (2005) have envisaged probiotics positively influencing systemic organs by modulating immune function, stimulating virus-specific antibody production, and affecting intestinal mucosa absorption and secretion (de Vrese and Schrezenmeir, 2002).

In preventing respiratory infections, children of the *L. casei rhamnosus* group had a reduction of 17% and 18% during the short- and long-term interventions, respectively, compared to the control group. Our current work was supported by a previous study showing that probiotics reduce respiratory infections and their severity among preschoolers (Hatakka et al., 2001). There was a reduction in the occurrence of recurrent respiratory infections in the multiple probiotic and the *L. rhamnosus* T cell-1 group, but it was insignificant (Hatakka et al., 2001). The diverse outcomes we observed among the three commercial probiotics may have

resulted from probiotic strain-dependent effectiveness. Previous investigations showed that when research subjects were given a mixture of probiotics, there was an insignificant effect in preventing the incidence of respiratory infections (de Vrese et al., 2006; Hatakka et al., 2007).

Together with increasing reports of clinical effects against infectious diseases, there is a growing interest of the role of probiotics in bacterial and viral infectious disease prevention. This large population study has successfully demonstrated that probiotics could induce differential effects upon infectious disease in preschoolers among the three orally administered commercial probiotics. However, the benefits of probiotics were small in reducing the incidence of disease in some subgroups. Various probiotics can be efficient immune modulators whose effectiveness varies among strains and species, such as *Lactobacillus* and *Bifidobacterium*. Our findings exhibit similarities to studies showing that *Lactobacilli* species can affect antigen-specific IgG1/ IgG2 Ab and cytokine responses (Maassen et al., 2003; Maassen et al., 2000). Certain strains of *Lactobacilli* can activate myeloid dendritic cells to stimulate T cells and then induce Th1 cytokines, and could be useful for the delivery of bio-therapeutic agents (Mohamadzadeh et al., 2005). This investigation strongly suggests that there is a need for rational probiotic selection and detailed evaluation prior to application in food or health care products. It also implies that the bacterial growth phase is a crucial parameter allowing for additional manipulation of immune responses by oral administration of *Lactobacilli*. A larger scale of investigation will be required to obtain more information about the effect of these parameters upon a study population.

In conclusion, this randomized, double-blind study shows that bio-therapeutic agents may be useful in preventing viral and bacterial infectious disease. However, different

commercial probiotics have dissimilar effects on diverse infectious disease. The *L. casei rhamnosus* strain may reduce most infectious diseases, especially respiratory infections. Multiple probiotic supplementation may significantly reduce gastrointestinal disease, and long-term consumption of *L. rhamnosus* T cell-1 could decrease the incidence of bacterial infection.

Author's contributions

JSL contributed to the design of the study and the questionnaires, and participated in creating the database and execution of data analysis. YHC performed the experimental assays and analysis, data analysis, prepared and wrote the manuscript and contributed to the discussion. NTL, CHC and KCH participated in the planning of the study and revised the manuscript. KCP and KWL conceived the study and contributed to its design, coordination, and supervision, and to the manuscript discussion and conclusions.

Acknowledgements

We thank the preschool staff, the children, and their parents for making this study possible.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported and funded by Success Medical Corporation, Ltd., Taipei, Taiwan; Chang Gung Biotechnology Corporation, Taipei, Taiwan; and Multipower Enterprise Corporation, Taipei, Taiwan.

FIGURES AND LEGENDS

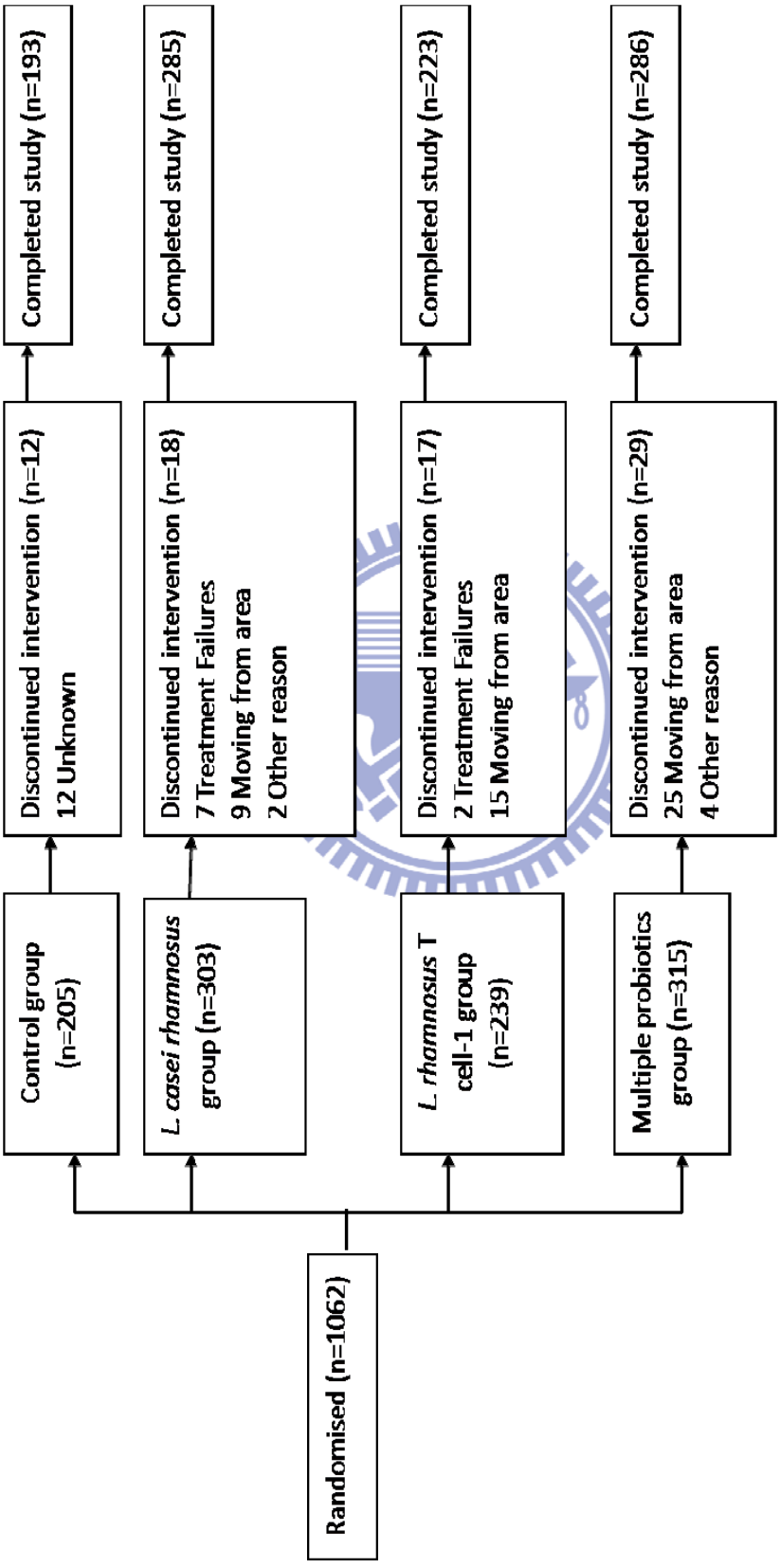

Figure 1. Flow chart showing progress of the participants through the trial.

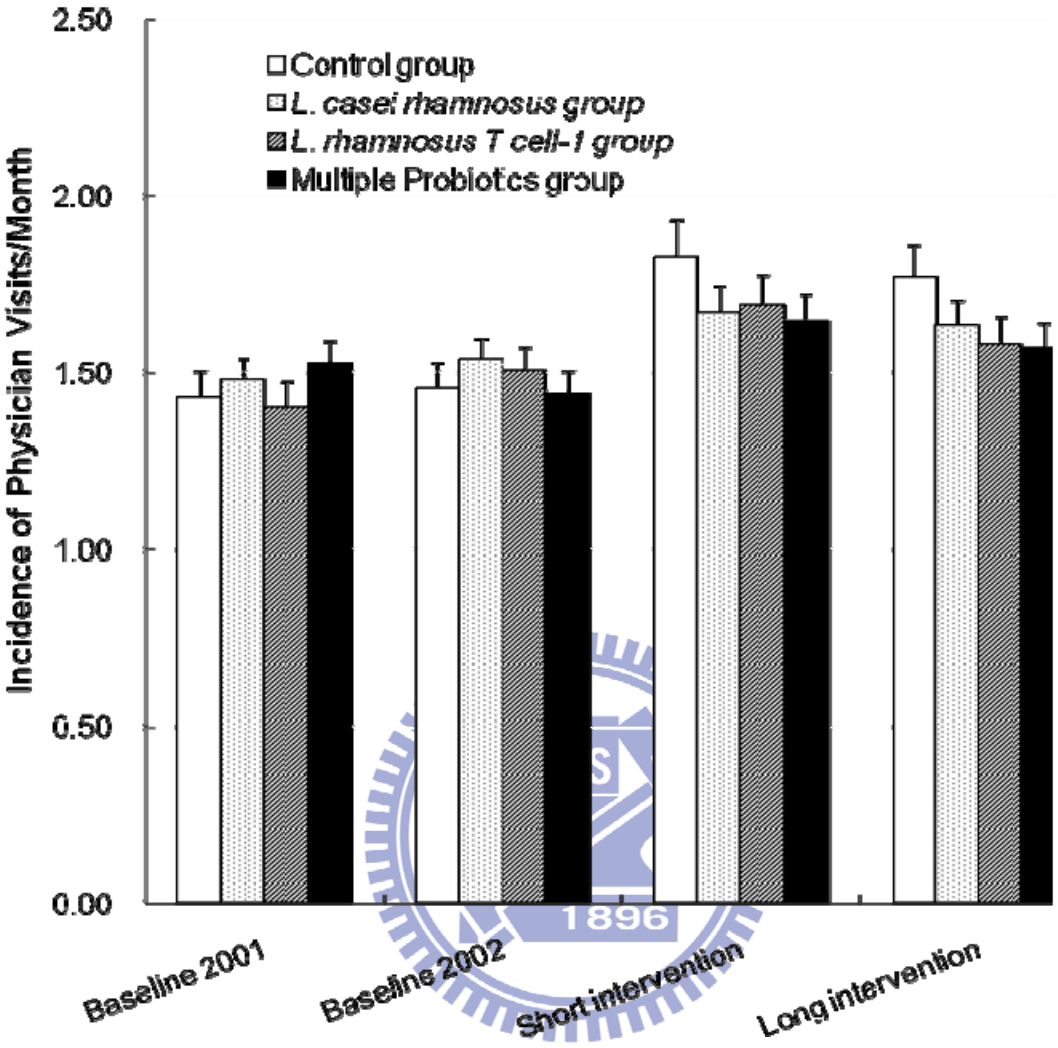
2.50
2.00
1.50
1.00
0.50
0.00
Incidence of Physician Visits/Month
Control group
L. casei rhamnosus group
L. rhamnosus T cell-1 group
Multiple Probiotics group
Baseline 2001
Baseline 2002
Short intervention
Long intervention

Figure 2. Effects of oral administration of the three different commercial probiotics on physician visits in preschool children.

Mean number of physician visits per month during the baseline period and intervention period in preschool children that received *L. casei rhamnosus* (⊡), *L. rhamnosus* T cell-1 (▨) or the multiple probiotic (■), compared to the control group (□). Baseline 2001 and 2002: Period before entrance to preschool and treatment of children allocated to the probiotic and control groups. Short-term: Period that the children had been treated with different commercial probiotics in the first 3.3 months. Long-term: Period that the children had been treated with different commercial probiotics during the whole 7.3 months.

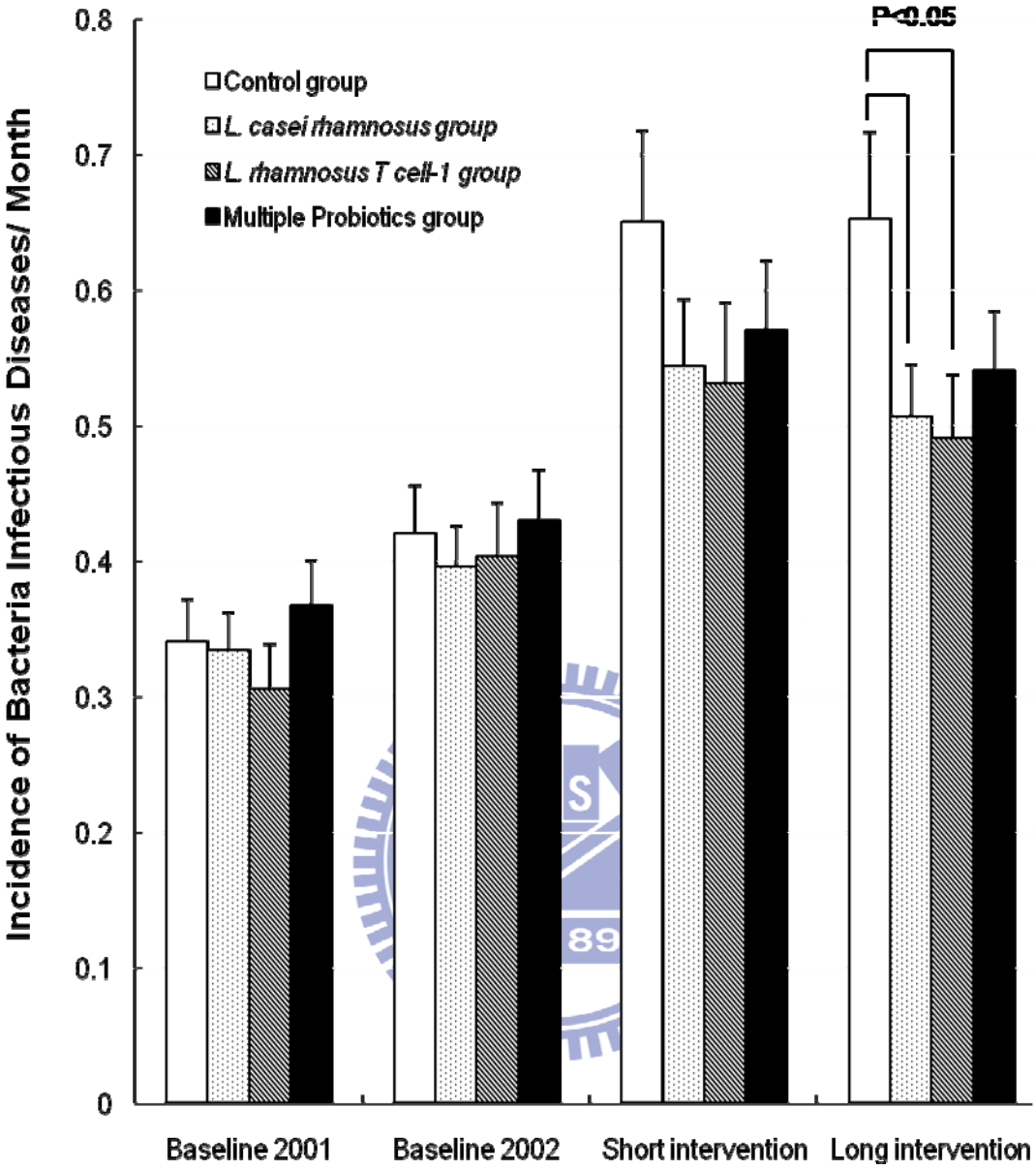
P<0.05
Control group
L. casei rhamnosus group
L. rhamnosus T cell-1 group
Multiple Probiotics group
Incidence of Bacteria Infectious Diseases/ Month
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Baseline 2001
Baseline 2002
Short intervention
Long intervention

Figure 3. Effects of the oral administration of the three different commercial probiotics on bacterial infectious disease in preschool children.

Mean number of bacterial infectious diseases per month during the baseline period and intervention period in preschool children that received *L. casei rhamnosus* (⬚), *L. rhamnosus* T cell-1 (▨) or the multiple probiotic (■), compared to the control group (☐). * Significantly different from the control group ($P<0.05$).

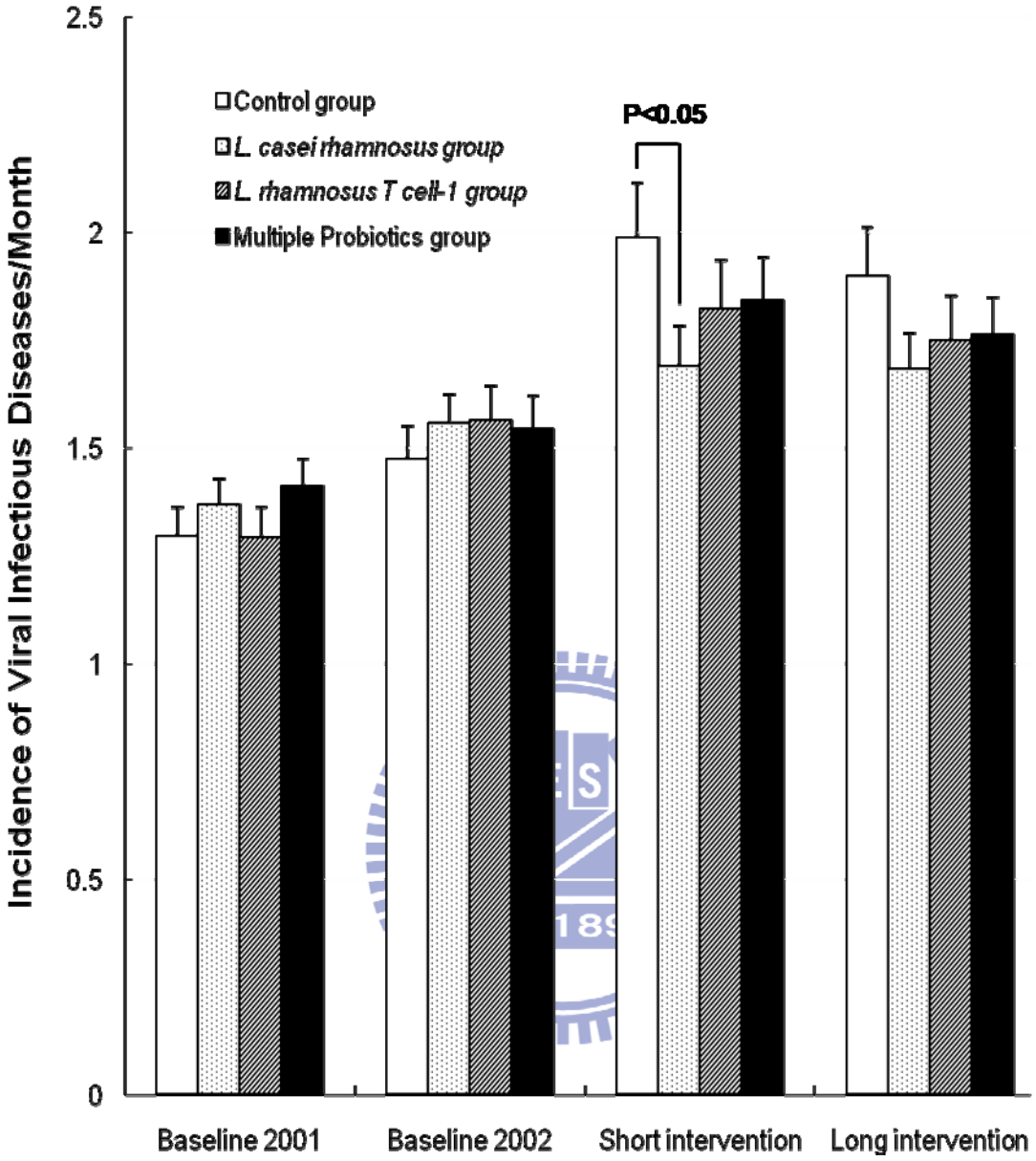
Incidence of Viral Infectious Diseases/Month
2.5
2
1.5
1
0.5
0
Control group
L. casei rhamnosus group
L. rhamnosus T cell-1 group
Multiple Probiotics group
P<0.05
Baseline 2001
Baseline 2002
Short intervention
Long intervention

Figure 4. Effects of the oral administration of the three different commercial probiotics on viral infectious disease in preschool children.

Mean number of viral infectious diseases per month during the baseline period and intervention period in preschool children that received *L. casei rhamnosus* (⬚), *L. rhamnosus* T cell-1 (▨) or the multiple probiotic (■), compared to the control group (☐). * Significantly different from the control group (P<0.05).

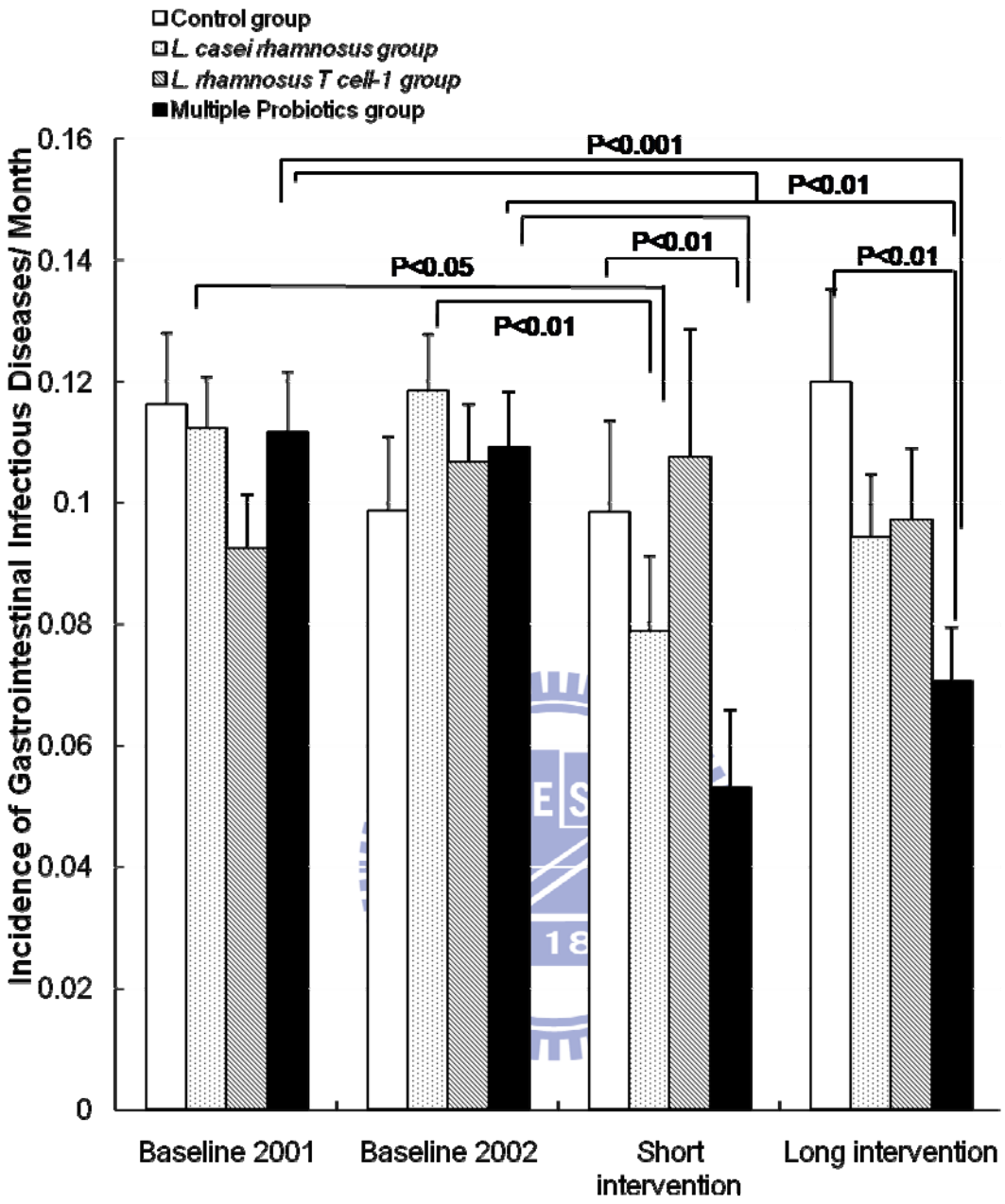
Control group
L. casei rhamnosus group
L. rhamnosus T cell-1 group
Multiple Probiotics group
Incidence of Gastrointestinal Infectious Diseases/ Month
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
P<0.001
P<0.01
P<0.01
P<0.01
P<0.05
P<0.01
Baseline 2001
Baseline 2002
Short intervention
Long intervention

Figure 5. Effects of oral administration of the three different commercial probiotics on gastrointestinal infectious disease in preschool children.

Mean number of gastrointestinal infectious diseases per month during the baseline period and intervention period in preschool children that received *L. casei rhamnosus* (⬚), *L. rhamnosus* T cell-1 (▨) or the multiple probiotic (■), compared to the control group (□). ** Significantly different from the control group ($P<0.01$).

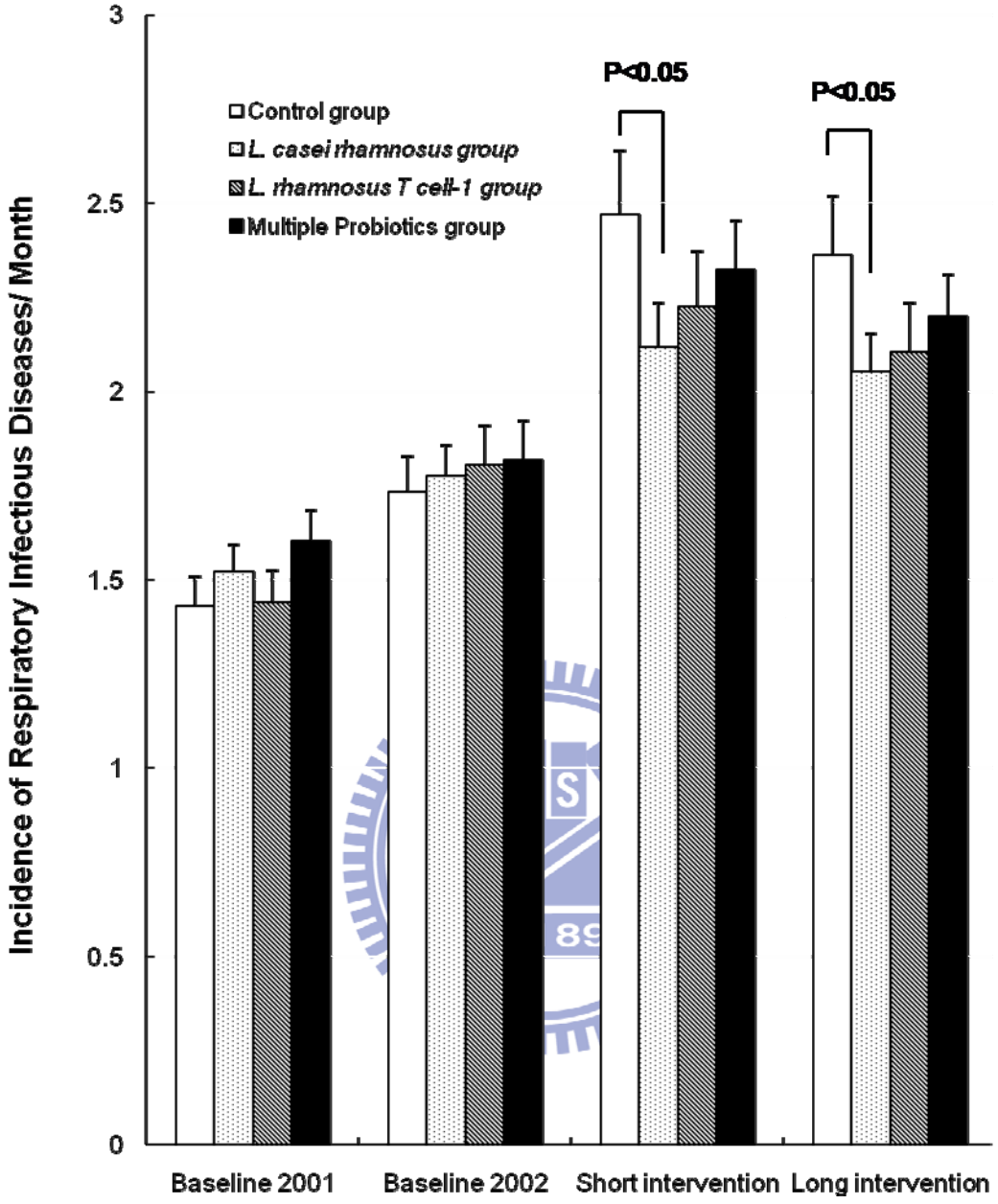
Incidence of Respiratory Infectious Diseases/ Month
3
2.5
2
1.5
1
0.5
0
Control group
L. casei rhamnosus group
L. rhamnosus T cell-1 group
Multiple Probiotics group
P<0.05
P<0.05
Baseline 2001
Baseline 2002
Short intervention
Long intervention

Figure 6. Effects of oral administration of the three different commercial probiotics on respiratory infectious disease in preschool children.

Mean number of respiratory infectious diseases per month during the baseline period and intervention period in preschool children that received *L. casei rhamnosus* (⬚), *L. rhamnosus* T cell-1 (▨) or the multiple probiotic (■), compared to the control group (□). * Significantly different from the control group (P<0.05).

Table 1. Baseline characteristics of the study groups

Characteristic	Control group (n=193)	*L. casei rhamnosus* group (n=285)	*L. rhamnosus* T cell-1 group (n=222)	Multiple probiotic group (n=286)
Age (years)	4.74±1.07	4.54±1.04	5.16±1.05	4.64±0.95
Male/Female	1.31	1.23	1	1.07
Duration of breast feeding (months)	1.51±3.35	1.73±3.89	1.62±3.84	2.39±5.01
House area (m^2)	44.3±20.2	40.4±20.1	43.9±21.0	50.3±25.38
Smoking in household	57%	63%	65%	55%
Family economic (10^4NT/year)	66.5±32.2	69.0±34.2	65.9±32.5	71.8±40.8
History of allergy (diagnosed by doctor)				
Asthma	4%	6%	5%	4%
Allergic rhinitis	15%	20%	18%	13%

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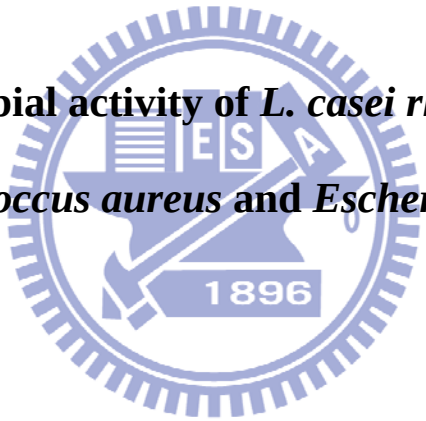


Innate antimicrobial activity of *L. casei rhamnosus* against *Staphylococcus aureus* and *Escherichia Coli*

ABSTRACT

It is well-known that probiotics have important roles for the health of the host including producing antimicrobial metabolites. The aim of this study was to determine the antimicrobial activities among *Lactobacillus* strains and coclutured with various pathogens. Antimicrobial activity of each supernatant from *Lactobacillus* strains, *L. casei rhamnosus*, *L. casei* and *L. rhamnosus*, were determined by radial diffusion test. Coculture with three kinds of pathogen (*Escherichia coli* ATCC 25922, *Clostridium difficile*, and *Bacteroides frigilis*) as inducers were carried out. The ability of *Lcr* supernatants were investigated to produce inhibition for *E. coli* ATCC25299 (as experimental model in gram-negative bacteria) and *Staphylococcus aureus* ATCC10780 (as experimental model in gram-positive bacteria) by radial diffusion test. *Lcr* promoted the greatest antimicrobial activities, producing inhibition halos corresponding to 20.75 and 20.25 mm for *E. coli* and *S. aureus*, respectively. There were no significant differences on the size of the inhibition halos in coculture *Lcr* with different pathogens or *Lcr* culture alone. The results indicated that pathogens cannot enhence the antimicrobial capacities of the *Lcr* strains. The antimicrobial capacities of *Lcr* are not pathogenesis-inducible.

INTRODUCTION

Lactic acid bacteria (LAB) including *Lactobacilli*, when administered in adequate amounts, have been known to confer a health benefit on the host, are referred to as probiotic strains (FAO/WHO, 2001). *Lactobacilli* are natural components of gastrointestinal flora recognized as beneficial to the host. The effectiveness of *Lactobacilli* is strain-specific that contributes to host health through different mechanisms. *Lactobacilli* can prevent or inhibit the proliferation of pathogens by antimicrobial substances (Sliva et al., 1987), suppress production of virulence factors by pathogens, or modulate the immune response. Antimicrobial components produced by *Lactobacilli* include organic acids, hydrogen peroxide, carbon dioxide, diacetyl, bacteriocins, and low molecular weight antimicrobial substances such as *reuterin* (Ouwehand and Vesterlund 2004).

Clinical studies examined standard antibiotic therapy with concurrent probiotic *Sacchraromyces boulardii* or placebo in 124 adult patients, 64 patients with an initial episode of *Clostridium difficile* disease and 60 patients with a history of at least one prior episode of *C. difficile* disease (McFarland et al., 1994). The patients with prior *C. difficile* disease, *S. boulardii* significantly inhibited further recurrences of disease. Recurrences prevention of *C. difficile*-associated diarrheas by *L. plantarum* (299v) has been tested in a small, boubled-blind,

placebo-controlled study (Wullt et al., 2003). Meanwhile, the antimicrobial activity of the intraurethrally administered probiotic *L. casei* strain Shirota against *Escherichia coli* in a murine urinary tract infection (UTI) model was examined. The results showed that a single administration of *L. casei* Shirota at a dose of 10^8 colony forming unit (CFU) 24 h before the challenge infection dramatically inhibited *E. coli* growth and inflammatory responses in the urinary tract. Multiple daily treatments with *L. casei* Shirota during the post-infection period also showed antimicrobial activity in this UTI model. *In vitro* studies also suggested multiple specific activates of different probiotic agents against several pathogens, including *Staphylococcus aureus* (Lahtinen et al., 2007) and *E. coli* (Mack et al., 1999).

Antimicrobial compounds produced by LAB have been widely studied, but it is questionable that different pathogen can effectively stimulate the various production of antimicrobial substances by *Lactobailli*. A previous study demonstrated that *L. casei rhamnosus* (*Lcr*) can control bacterial and gastrointestinal disease (Lin et al., 2009), which motivated this investigation on the effectiveness of *Lcr* on antimicrobial effects. In the present study, antimicrobial activities of supernatants obtained from *Lactobacillus* strains cocultured with heat-killed pathogen strains were examined.

A previous study demonstrated that supernatant of *Lcr* with molecular weight of <5 kDa

exhibited best growth inhibitive ability against indicator strains. The antimicrobial activity from *Lcr* supernatants were not induced by heat-treated pathogenic bacteria implying antimicrobial components of *Lcr* being not bacteria-inducible.

MATERIALS AND METHODS

The *Lactobaciilus* strain, *L. casei rhamnosus* (*Lcr*), was obtained from Antibiophilus® Laboratoires Lyocentre Ltd, Aurillac, France. *L. casei,* and *L. rhamnosus* were provided by Tzu Chi General Hospital (Hualien, Taiwan). All *Lactobacillus* strains were grown at 37°C in MRS broth (Difco, Sparks,MD, USA) for 48 h until stationary phase and cell-free supernatants were collected by centrifugation at 1,000×*g* for 10 min at 4°C.

Cell-free supernatants of *Lactobacillus* strains were collected and centrifuged in a filtering device (Amicon Ultra-15; Millipore, Bedford, MA, USA) using MWCO 5 kDa ultrafiltration membranes. The supernatant was loaded into 5kDa ultrafiltration device, 3300×*g* for 30 minutes. The concentrate was the >5kDa fraction. The filtrate was the <5 kDa fraction. Each fraction was filtered through a 0.22 μm membrane (Millipore) and kept at 4°C before use. Protein concentration was determined by the BCA protein assay kit (Pierce, Rockford, IL, USA) following the manufacturer's instructions.

Staphylococcus aureus (ATCC 10780) and *Escherichia coli* (ATCC 25922) were obtained from the American Type Culture Collection (Rockville, Md.) and grown in Luria–Bertani (LB; Sigma-Aldrich Co. Ltd. Poole, UK). Solid media contained 1.5% (w/v) agar (Sigma-Aldrich). *Clostridium difficile* and *Bacteroides frigilis* were kindly supplied by

Tzu Chi General Hospital and cultured in an anaerobic atmosphere on TCCA plates (brain-heart infusion agar supplemented with 5% defibrinated forse blood, 0.1% taurocholate, 10mg mL^{-1} cefoxitin and 10mg mL^{-1} cycloserine) (Difco). Cells were washed twice with sterile distilled water, suspended in phosphate-buffered saline (PBS; 0.85% NaCl, 2.68 mM KCl, 10 mM Na_2HPO4, and 1.76 mM KH_2PO_4 at pH 7.7), and then heat-killed at 100 °C for 15 min.

Lactobacillus strains were treated with heat-killed cells of *E. coli, C. difficile* and *B. frigilis* at a ratio of 1:1, 1:10 and 1:100 respectively, and incubated at 37 °C for 48 h. Cell-free supernatants were collected by centrifugation at 1,000×*g* for 10 min at 4°C.

The radial diffusion test was performed as described previously (Coconnier et al., 1997). Briefly, 5×10^6 CFU per mL of *S. typhimurium* ATCC 10780 or *E. coli* ATCC 25922 were added to 10mL of Trypticase soy broth (TSB) (Sigma-Aldrich) or LB agar, respectively, and poured into a square Petri dish. The test material (25 μL) was applied in a well punched in the agar and incubated for 3 h at 37 °C. Subsequently, the plates were overlaid with 10mL of sterile TSB agar solution (1% agarose). The diameter of inhibition halo was measured after 18–24h (Sreeramulu et al., 2000). All determinations were performed in triplicate. Kanamycin (Sigma-Aldrich) at 50μM was used as a positive control and fresh MRS broth was used as a

negative control in antimicrobial activity experiments.

All experiments were performed at least 3 times, each time in triplicate. Data were analyzed by multivariate ANOVA test. If a significant difference was found, a least significant differences (LSD) multiple comparison test was used to identify significant groups. Statistical analyses used The Statistical Software Package for the Social Sciences, version 12.0.1 for Windows (SPSS Inc., Chicago, IL, USA). A P value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

To obtain an overview of the antimicrobial capacity of *Lactobacillus* strains, and to identify the specific strains capable of pathogen inhibition, *L. casei rhamnosus* (*Lcr*), *L. casei* (*Lc*) and *L. rhamnosus* (*Lr*) were investigated. The initial results indicated that all supernatants inhibited the growth of *E. coli* ATCC 25922 and *S. aureus* ATCC 10780, but *Lcr* showed statistically significant inhibition diameter of halo zones (20.75 ± 0.35 mm) for *E. coli* ATCC 25922 (Figure 1A and 1C) and for *S. aureus* ATCC 10780 (20.25 ± 1.77 mm; Figure 1B and 1D).

In the present study, several *Lactobacillus* strains were investigated for their differential antimicrobial capabilities for gram-negative bacteria (*E. coli* ATCC 25922) and gram-positive bacteria (*S. aureus* ATCC 10780). *Lcr* exhibited more potent growth inhibiting capability than the *L. casei* and the *L. rhamnosus* strains. Probiotics, as antimicrobial agents against pathogens, are an attractive, alternative approach for the attenuation of intestinal pathogens-associated diseases. Many clinical studies during recent decades demonstrated that probiotic species possessed beneficial effects for bacterial infections (Lin et al., 2009; McFarland et al., 1994). However, inconsistency among some results might have resulted from differences in probiotic species or strains. These inconsistencies have drawn attention to understanding the mechanisms of probiosis by specific strains for possible clinical

applications.

Based on published evidence that *Lactobacillus* spp. secretes low molecular weight components (<5 kDa) possessong antimicrobial activity (Ouwehand and Vesterlund 2004; Servin, 2004). It was determined whether the antimicrobial activity observed in this study arose from such molecules. Supernatants of *Lactobacillus* strains was divided into 2 fractions (<5 kDa and >5 kDa) based on molecular weight using ultrafiltration membranes. As illustrated in Fig. 1 we found that the filtrate retained most activity indicating that the active component has a molecular weight of <5 kDa. The diameter of halo zones of *Lcr* fractions in inhibition for *E. coli* ATCC 25922 and *S. aureus* ATCC 10780 were as follows: *Lcr* cell-free supernatant, 20.75 ± 0.35 mm and 20.25 ± 1.77 mm, respectively; <5 kDa fraction, 19.5 ± 2.12 mm and 20.75 ± 0.35 mm, respectively; >5 kDa fraction, 14 ± 1.41 mm and 16.75 ± 0.35 mm, respectively ($p<.05$). Both *Lc* and *Lr* strains were not as effective as the *Lcr*, suggesting that the inhibitory activity is a unique property even among known probiotic strains.

To examine whether antimicrobial activity would increase with pathogens challenge, we next stimulated *Lcr* with the serial tenfold various pathogens. After 48 h coculture, various *Lcr* supernatants were collected and antimicrobial activities were measured. As illustrated in Fig. 2, we found that there were no significant differences on inhibition diameter of halo

zones for *E. coli* ATCC 25922 from various pathogens or various ratios of pathogens stimulation compared with *Lcr* culture alone (15 mm). The diameter of halo zones of inhibition for *E. coli* ATCC 25922 from *Lcr* supernatants with 1-fold, 10-fold and 100-fold pathogens stimulation were as follows: *CD*, 14.75 ± 0.35 mm, 14 mm and 12.75 ± 1.06 mm, respectively; *B. frigilis*, 17.75 ± 0.35 mm, 16.5 mm and 12.75 ± 0.35 mm, respectively; *E. coli* ATCC 25922, 16 mm, 14.75 ± 0.35 mm and 14.25 ± 1.06 mm, respectively.

All supernatants had significant inhibitory effects on the growth of the gram-positive indicator strains of *S. aureus* ATCC 10780. In general, gram-positive strains are more capable of inhibiting other gram-positive strains than gram-negative strains. The diameter of halo zones of inhibition for *S. aureus* ATCC 10780 from *Lcr* supernatants with 1-fold, 10-fold and 100-fold pathogens stimulation were as follows: *CD*, 18 ± 0.7 mm, 16.25 ± 0.35 mm and 13.25 ± 1.76 mm, respectively; *B. frigilis*, 20.75 ± 1.06 mm, 18.5 mm and 16.75 ± 0.35 mm, respectively; *E. coli* ATCC 25922, 20 ± 0.7 mm, 18.75 ± 0.35 mm and 19.75 ± 0.35 mm, respectively.

There are different factors that can influence antimicrobial components production: pH (Kaiser and Montville, 1993; Cotter et al., 2005), temperature (Diep et al., 2000) and growth conditions (Biswas et al., 1991). However, the effect of the presence of competing

microorganisms on antimicrobial components production is not well known yet. Our findings exhibit dissimilarities to studies showing that antimicrobial components from *Lactobacillus* strains are bacteria-inducible. Rojo-Bezares et al. (2007) showed that an inducible antimicrobial component, bacteriocin, has been detected in *L. plantarum* strain J23. This antimicrobial activity was induced by living or heat-treated cells of some LAB strains and presents stability in a wide range of pHs and after high temperature treatments. In addition, Røssland et al. (2005) found that growth of LAB together with pathogen, *Bacillus cereus,* gives better conditions for the LAB, which results in greater quantities of different metabolites, especially antimicrobial components. A further investigation will be required to obtain more information about the inducible effect of these antimicrobial components.

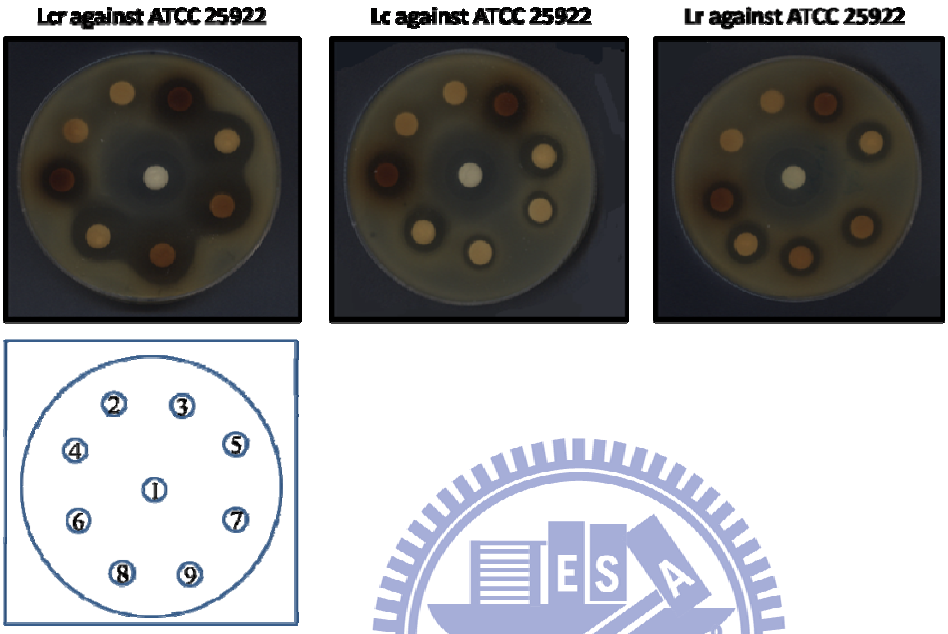
Lcr supernatants from different ratio of *CD* challenge were separated by molecular filtration using membranes with molecular weight cutoff of 5 kDa. All fractions showed antimicrobial effects on *E. coli* ATCC 25922 and *S. aureus* ATCC 10780. <5 kDa *CD* challenge fraction showed a stronger antimicrobial effect on indicator bacteria. Hence, it would seem that active components exist at <5 kDa fraction.

In conclusion, *Lcr* show best growth inhibitive ability against indicator strains, the active antimicrobial substances has a molecular weight of <5 kDa. The antimicrobial activity from

Lcr supernatants were not induced by heat-treated cells of some pathogenic bacteria, it was implied that antimicrobial components from *Lcr* are not bacteria-inducible. Our work is among the first studies focusing on the assessment of antimicrobial activity of *Lcr* strains, a useful species in clinical and nutritional fields. The results of this study should be assessed in association with colonization resistance of the microbiota, which may be modified not only by the antimicrobial properties of specific components of the microbiota, but also by the impact of *Lcr* on the composition and metabolic.

FIGURES AND LEGENDS

A.



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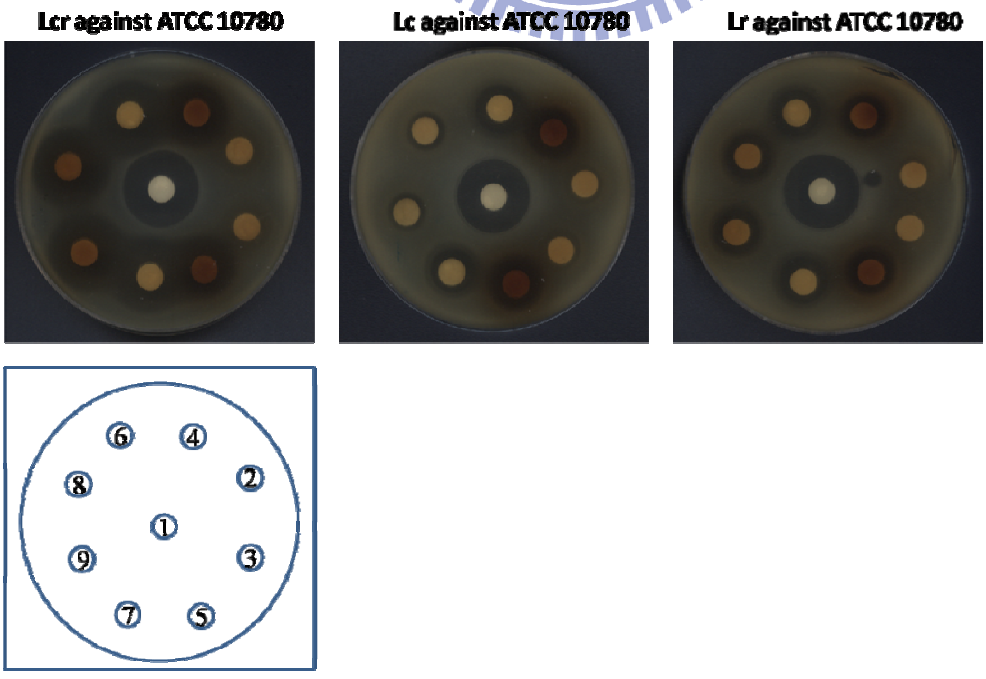

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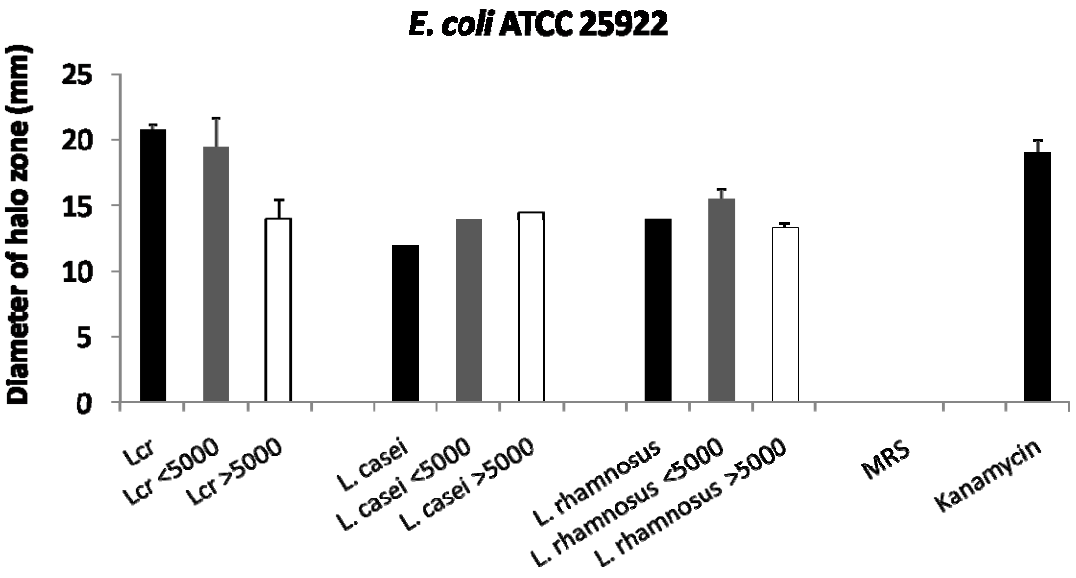

E. coli ATCC 25922
Diameter of halo zone (mm)
25
20
15
10
5
0
Lcr
Lcr <5000
Lcr >5000
L. casei
L. casei <5000
L. casei >5000
L. rhamnosus
L. rhamnosus <5000
L. rhamnosus >5000
MRS
Kanamycin

D.

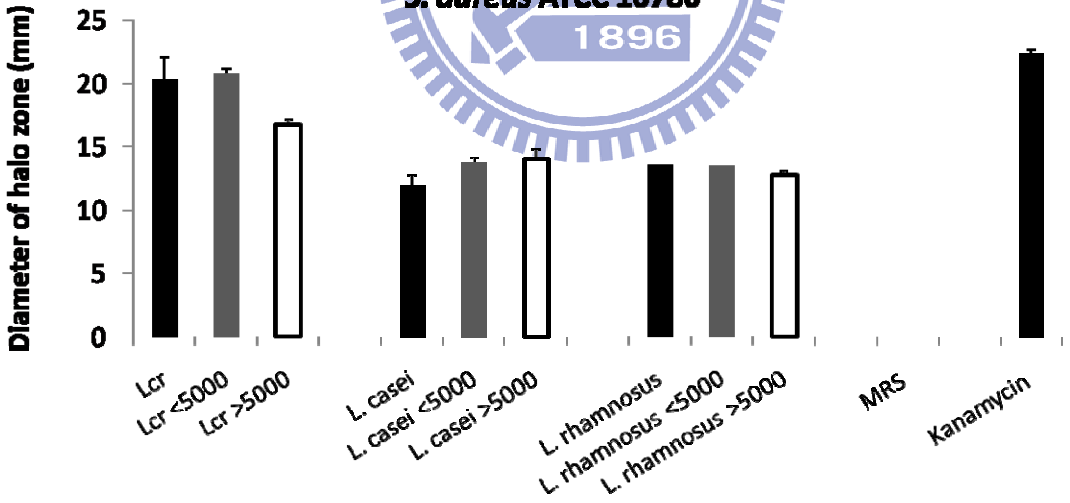
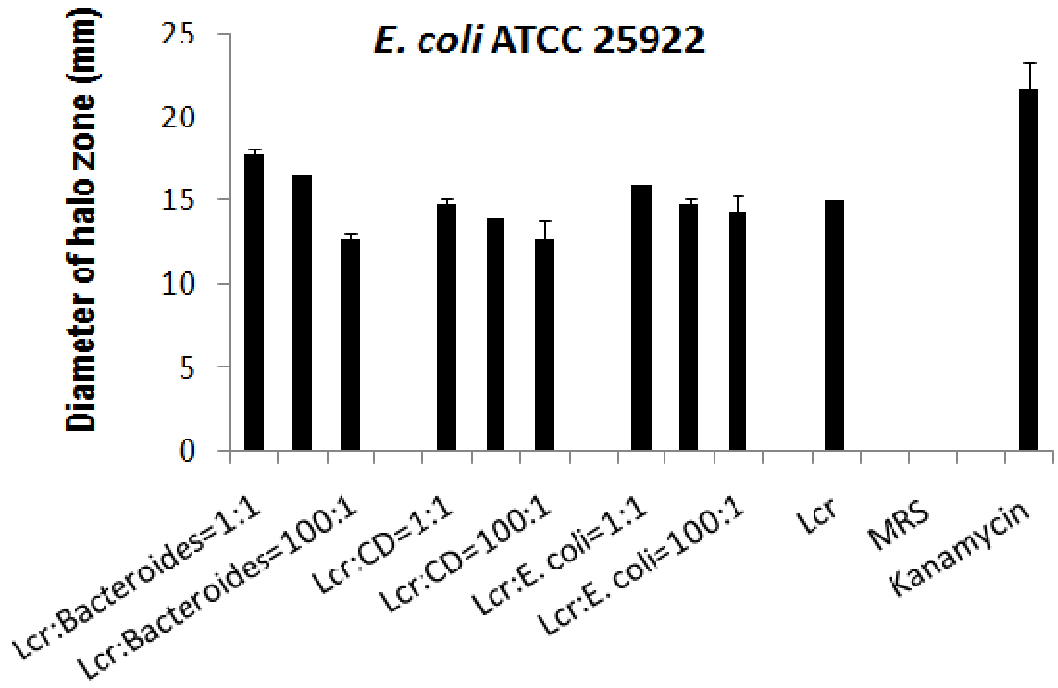

S. aureus ATCC 10780
Diameter of halo zone (mm)
25
20
15
10
5
0
Lcr
Lcr <5000
Lcr >5000
L. casei
L. casei <5000
L. casei >5000
L. rhamnosus
L. rhamnosus <5000
L. rhamnosus >5000
MRS
Kanamycin

Figure 1. Antimicrobial activity of supernatant of *Lactobacillus* strains

Data from *E. coli* ATCC 25922 and *S. aureus* ATCC 10780 exposed to *Lactobacillus* spp. supernatants that had been passaged through a <5 Kda molecular mass cut-off filter. Cell-free supernatant (■), <5 kDa fraction of supernatant (■) or >5 kDa fraction of supernatant (□) were collected after incubation with *L. casei rhamnosus* (*Lcr*), *L. casei* (*Lc*) or *L. rhamnosus* (*Lr*) for 48hrs. 25 μL of each supernatant was applied for inhibition for *E. coli* ATCC 25922 (panel A and C) or for inhibition for *S. aureus* ATCC 10780 (panel B and D). The diameter of inhibition halo was measured after 18–24h. Results representative of typical donors (panel A and B) and mean ± standard error of the mean (panel C and D) for 3 different experiments. Kanamycin (Sigma-Aldrich) at 50μM was used as a positive control and fresh MRS broth was used as a negative control in antimicrobial activity experiments. ***P< 0.001 for change versus *Lcr* supernatant.

Schematic description of the plate antimicrobial assay from different supernatants of *Lactobacillus* strains. Small circles represent various supernatants treatment on *E. coli* ATCC 25922 (A) or *S. aureus* ATCC 10780 (B) growing agar medium in a Petri plate (large circles). (1) Kanamycin; (2) and (3) MRS; (4) and (5) cell-free supernatant; (6) and (7) <5 kDa fraction; (8) and (9) >5 kDa fraction.

A.


E. coli ATCC 25922
Diameter of halo zone (mm)
25
20
15
10
5
0
Lcr:Bacteroides=1:1
Lcr:Bacteroides=100:1
Lcr:CD=1:1
Lcr:CD=100:1
Lcr:E. coli=1:1
Lcr:E. coli=100:1
Lcr
MRS
Kanamycin

B.

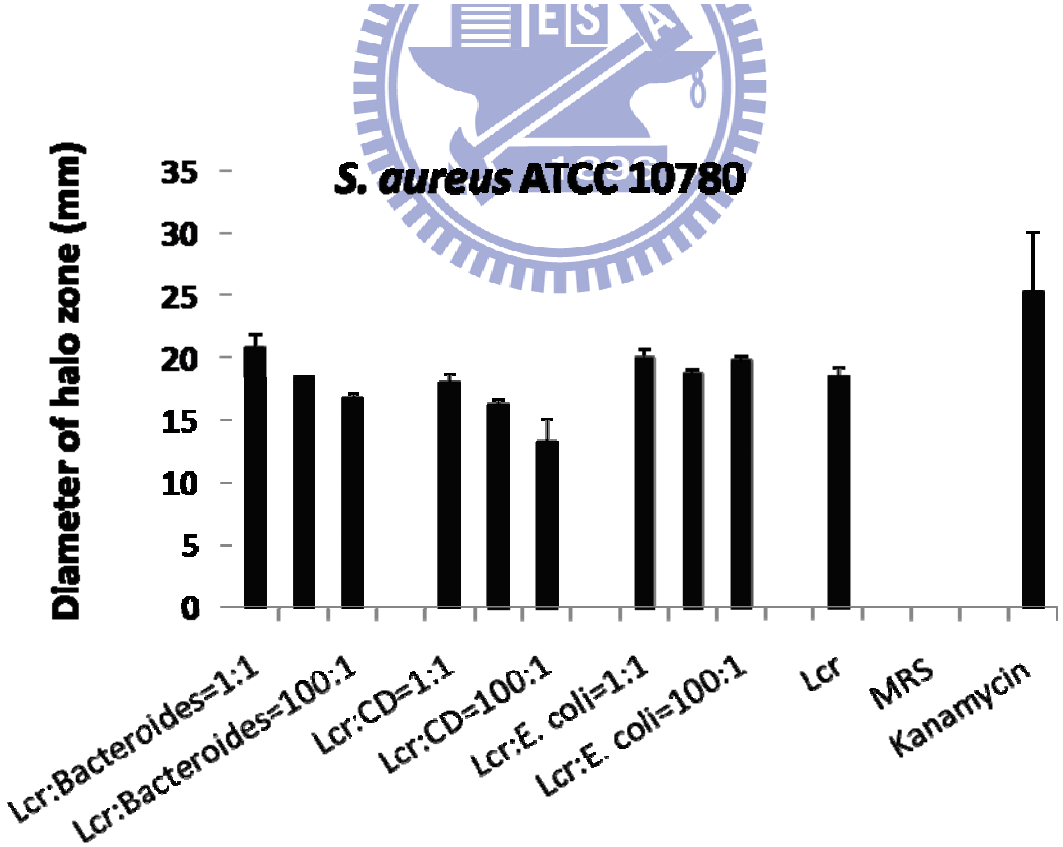
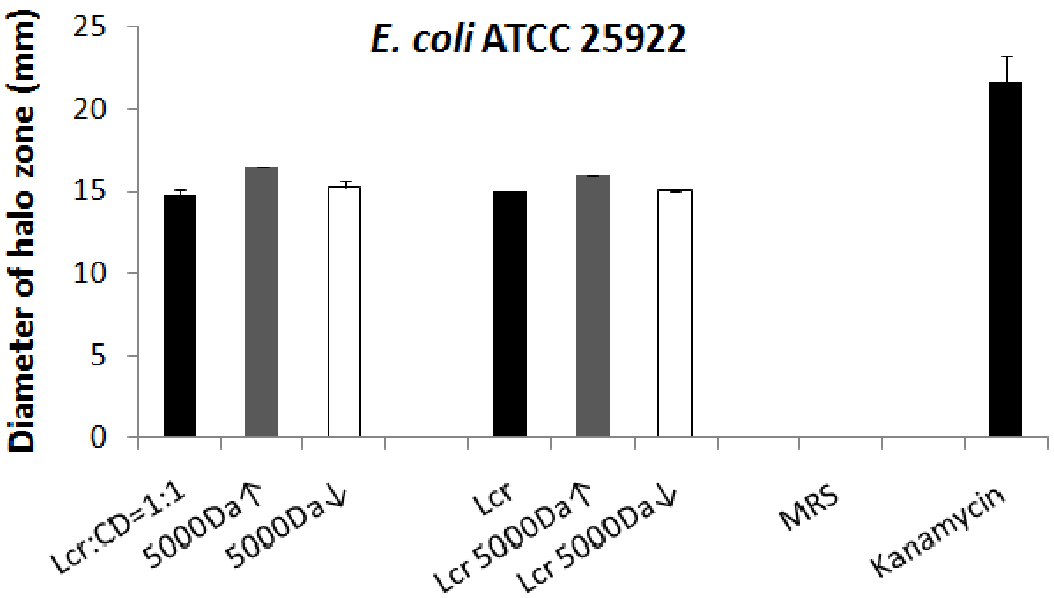

S. aureus ATCC 10780
Diameter of halo zone (mm)
35
30
25
20
15
10
5
0
Lcr:Bacteroides=1:1
Lcr:Bacteroides=100:1
Lcr:CD=1:1
Lcr:CD=100:1
Lcr:E. coli=1:1
Lcr:E. coli=100:1
Lcr
MRS
Kanamycin

Figure 2. Antimicrobial activity of *Lcr* stimulated with various heat killed pathogens

Inhibition activity for *E. coli* ATCC 25922 (panel A) or for inhibition for *S. aureus* ATCC 10780 (panel B) derived from *Lcr* to stimulated with various heat killed pathogen, *C. difficile* (*CD*), *Bacteroides frigilis* and E. coli ATCC 25922. Supernatants were collected after 48hrs cultured at 37^{o}C and stimulations of *Lcr* were done at various ratios of 1:1, 1:10 and 1:100 (*Lcr*/pathogen). The diameter of inhibition halo was measured after 18–24h. Kanamycin (Sigma-Aldrich) at 50μM was used as a positive control and fresh MRS broth was used as a negative control in antimicrobial activity experiments. Results are mean ± standard error of the mean of 3 individual experiments. ***P< 0.001 for change versus supernatant of *Lcr* cultured alone.

A.


E. coli ATCC 25922
Diameter of halo zone (mm)
25
20
15
10
5
0
Lcr:CD=1:1
5000Da↑
5000Da↓
Lcr
Lcr 5000Da↑
Lcr 5000Da↓
MRS
Kanamycin

B.

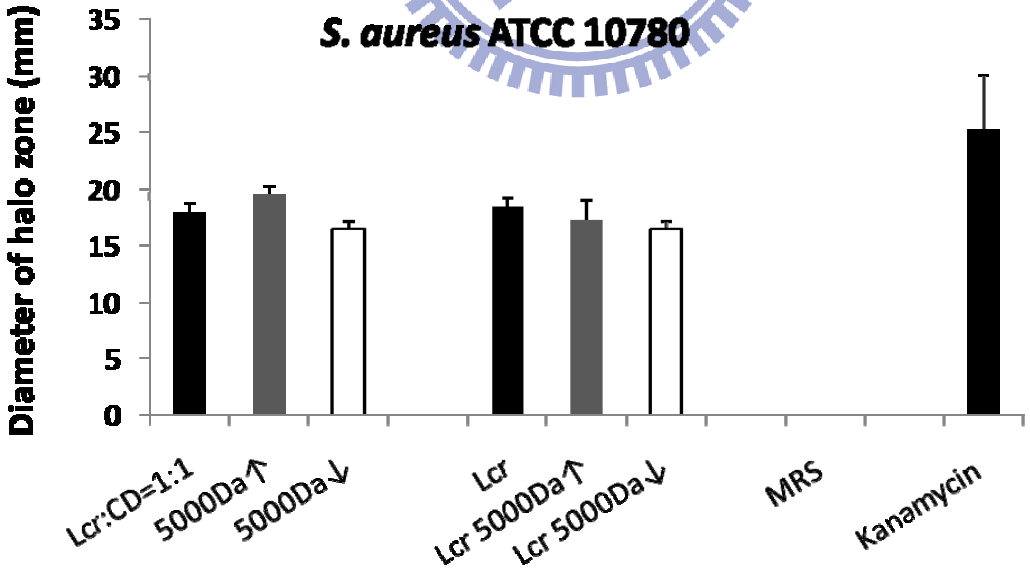

S. aureus ATCC 10780
Diameter of halo zone (mm)
35
30
25
20
15
10
5
0
Lcr:CD=1:1
5000Da↑
5000Da↓
Lcr
Lcr 5000Da↑
Lcr 5000Da↓
MRS
Kanamycin

Figure 3. Antimicrobial activity from different fractions of *Lcr* supernatant stimulated with heat killed *C. difficile*

Cell-free supernatant (■), <5 kDa fraction of supernatant (■) or >5 kDa fraction of supernatant (□) were collected after incubation with various ratio of *Lcr* and *CD* coculture supernatants. 25 μL of each supernatant was applied for inhibition for *E. coli* ATCC 25922 (panel A) or for inhibition for *S. aureus* ATCC 10780 (panel B). The diameter of inhibition halo was measured after 18–24h incubation. Kanamycin (Sigma-Aldrich) at 50μM was used as a positive control and fresh MRS broth was used as a negative control in antimicrobial activity experiments. Results are mean ± standard error of the mean of 3 individual experiments. ***$P < 0.001$ for change versus *Lcr* supernatant.

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Preferential promotion of apoptosis of monocytes by *Lactobacillus casei rhamnosus* soluble factors

ABSTRACT

Inflammatory bowel disease (IBD) is characterized by dense infiltrates of and defective apoptosis by mucosal cell populations. Some probiotics inhibit monocytes' expansion, although mechanisms remain unknown. Supernatants of *Lactobacillus* strains were investigated for inducing apoptosis of monocytes. Secreted factors produced by *Latobacillus* strains were tested on human lymphocytes, monocytes and a human monocytic leukemia- cell line (THP-1). Cell death mechanisms were investigated by a variety of methods. Lipopolysaccharide (LPS) - induced proinflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α) and anti-inflammatory TGF-β1 were determined. Soluble factor(s) from *L. casei rhamnosus* strain supernatants (*LcrS*) effectively induced apoptosis of immune cells. These were mainly soluble proteins (MW 5-30 kDa; *Lcr*$S_{5\text{-}30}$). For immune cells, but not human colonic epithelial carcinoma cells (HT-29), pretreatment with *Lcr*$S_{5\text{-}30}$ significantly promoted apoptosis via a mitochondrial pathway. *Lcr*$S_{5\text{-}30}$ suppressed pro-inflammatory cytokines and induced anti-inflammatory TGF-β1. Probiotic *Lcr* produced heat-stable molecules (MW range 5-30 kDa) that promoted immune cell apoptosis without affecting intestinal epithelial cells. *Lcr*$S_{5\text{-}30}$ triggered apoptosis by a mitochondrial pathway, but not via TGF-β signaling pathway. *Lcr*$S_{5\text{-}30}$ also inhibited LPS-induced inflammatory cytokines by immune cells. Thus, *Lcr*$S_{5\text{-}30}$ promotes apoptosis of immune cells, and suggests probiotics-based

regimens for prevention of IBD.

Keywords: *L. casei rhamnosus*, apoptosis, monocytes, IBD, mitochondrial pathway, TGF-β

INTRODUCTION

Probiotics have been evaluated as an alternative and safe therapeutic approach for inflammatory bowel disease (IBD) (Bibiloni et al., 2005). Clinical trials suggested that treatments with a multi-species probiotic, VSL#3, including 3 *Bifidobacterium* species, 4 *Lactobacillus* species and *S. salivarius* ssp. *thermophilus*, were effective for maintaining remission and decreasing relapse rates in IBD patients (Miele et al., 2009). Treatment using *Escherichia coli* Nassle 1917 showed remission maintenance for ulcerative colitis (UC) (Kruis et al., 2004). However, conflicting results have been observed for various probiotic strains in clinical use. Treatment with either *L. rhamnosus GG* (LGG) or *L. johnsonii* (LA1) did not improve clinical conditions for Crohn's disease (CD) or prevent endoscopic recurrence of CD, respectively (Marteau et al., 2006; Bousvaros et al., 2005).

The precise molecular mechanisms by which particular probiotics improve IBD remain unknown. Recent studies suggested that several probiotic strains could have apoptosis inducing capabilities, which would have positive effects on inflammatory responses. *E. coli* Nissle 1917 supernatant induced γδ T cell apoptosis via caspase- and FasL-dependent pathways (Guzy et al., 2008). In contrast, *L. reuteri* secreted factors promoted human myeloid leukemia-derived cells

apoptosis by modulation of NF-κB and MAPK signaling in the presence of TNF-α (Iyer et al., 2008). In addition, soluble proteins produced by LGG can regulate intestinal epithelial cell proliferation and survival by preventing cytokine-induced apoptosis (Yan et al., 2007). These studies illustrated diverse protective effects by different probiotic strains on inflammatory diseases.

IBD is a chronic recurrent inflammatory condition of the gastrointestinal tract. Although its etiology remains unknown, dense infiltrates of activated T cells and macrophages in the terminal ileum and colon mucosa are characteristics of IBD (Lügering et al., 2006). Defective apoptosis of mucosal T cells, macrophages and monocytes has been recognized as either an initiating event or necessary step in the pathogenesis of IBD (Itoh et al., 2001). Highly apoptosis-resistant mucosal cells may trigger inflammation, resulting in increased production of inflammatory cytokines (TNF-α, IL-1β, IL-8, etc.), cytolytic enzymes and reactive oxygen species causing tissue injury and clinical consequences (McGuckin et al., 2008; Strober et al., 2002). Various pharmaceutical treatments, including corticosteroids, antibiotics and immunomodulators, have been examined regarding their capacity to induce apoptosis in gut mucosal effector cells in IBD (Lügering et al., 2006). However, current therapeutic agents are only moderately effective for long-term treatment and have been associated with potential long-

term toxicity (Baert et al., 2003). Thus, alternative strategies for treatments of IBD have been devised to resolve adverse effects and to improve the clinical efficacy.

A previous study demonstrated that *Lcr* can control bacterial and gastrointestinal disease (Lin et al., 2009), which motivated this investigation on the effectiveness of *Lcr* on host immune mediation. Using human purified monocytes, this study showed the potency of *Lcr* promoted apoptosis of lymphocytes, monocytes and human monocytic leukemia-derived cell lines (THP-1). *Lcr* promoted selected cells apoptosis via a mitochondrial pathway and was strain-specific. In the presence of protein factors (MW 5-30 kDa), *Lcr* suppressed the production of pro-inflammatory cytokines by *E. coli* lipopolysaccharide (LPS)-activated lymphocytes, monocytes and THP-1 cells independent of apoptosis. Moreover, *Lcr*$S_{5\text{-}30}$ could induce the production of anti-inflammatory TGF-β1 and TGF-β-independent cell death.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The *Lactobaciilus* strain, *L. casei rhamnosus* (*Lcr*), was obtained from Antibiophilus® Laboratoires Lyocentre Ltd, Aurillac, France. *L. casei,* and *L. rhamnosus* were provided by Tzu Chi General Hospital (Hualien, Taiwan). All *Lactobacillus* strains were grown at 37°C in MRS broth (Difco, Sparks,MD, USA) for 48 h until stationary phase and cell-free supernatants were collected by centrifugation at 1,000×*g* for 10 min at 4°C. Fresh MRS broth was used as a control in apoptosis experiments and the cytokine assays.

Bacterial supernatant collection and size exclusion assays

Cell-free supernatant was collected and centrifuged in a filtering device (Amicon Ultra-15; Millipore, Bedford, MA, USA) using MWCO 50 kDa, 30 kDa and 5 kDa ultrafiltration membranes. The supernatant was loaded into 50 kDa ultrafiltration device, 3300×*g* for 30 minutes. The concentrate was the >50 kDa fraction. The <50 kDa fraction was centrifuged at 3300×*g* for 30 min using a 30 kDa ultrafiltration membrane. The upper layer was the 30-50 kDa fraction. The 5-30 kDa fraction was obtained by applying the <30 kDa fraction to 5 kDa ultrafiltration device. The filtrate was the <5 kDa fraction. Each fraction was filtered through a

0.22 μm membrane (Millipore) and kept at 4°C before use. Protein concentration was determined by the BCA protein assay kit (Pierce, Rockford, IL, USA) following the manufacturer's instructions.

Denaturation of bacterial supernatant proteins

The supernatant was mixed with 1 mg/ml trypsin or proteinase K (Sigma-Aldrich Co. Ltd. Poole, UK) at room temperature overnight. The supernatant was heated to 100 °C for 30 minutes prior to an intended assay.

Cell culture

THP-1 cells (human monocytic leukemia- derived cell line) and HT-29 cells (human colonic epithelial carcinoma cell line) were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). THP-1 cells were maintained in Falcon 100 × 20mm plastic culture flasks (Becton Dickinson Labware, Oxnard, CA, USA) in RPMI-1640 medium (Gibco, Invitrogen, Paisley, UK), supplemented with 10% fetal bovine serum (Gibco, Invitrogen), L-glutamine (200 mmol/l), 2-mercapto-ethanol (5×10^{-5} mol/l) (Sigma-Aldrich), 0.1 mg/ml of streptomycin sulfate (Sigma-Aldrich), 0.1 mg/ml of penicillin (Brittania Pharmaceuticals Ltd., Poole, UK). HT-29 cells were grown in Dulbecco's modified Eagle's medium (Gibco, Invitrogen)

supplemented with 10% fetal bovine serum. All cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2 for varying times.

Isolation PBMCs and purification of lymphocytes and monocytes

Peripheral blood mononuclear cells (PBMCs) obtained from healthy blood donors and were prepared by density gradient centrifugation of blood over Histopaque-1077 (Sigma-Aldrich). PBMCs were separated into CD14+ monocytes and lymphocyte subsets by immunomagnetic selection with CD14 MicroBeads kit (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany) used according to the manufacturer's instructions. Flow cytometry analysis indicated the purity of monocyte preparation from PBMCs following immunomagnetic selection with anti-CD14 was >95%. A highly enriched population of lymphocytes was obtained from the CD14-depleted cell fraction. Cell preparations were aliquoted to 1×10^6 cells/ml in RPMI 1640 medium (Gibco, Invitrogen) supplemented with 10% fetal calf serum (Gibco Invitrogen), 0.1 mg/ml of streptomycin sulfate (Sigma-Aldrich), 0.1 mg/ml of penicillin (Brittania Pharmaceuticals Ltd) and 100 mM L-glutamine (Sigma-Aldrich). Cell culture was at 37°C in a humidified atmosphere with 5% CO_2 for varying times.

Annexin V staining

Lymphocytes, monocytes and THP-1 cells (10^6 per test) were collected after incubation with *Lcr*S, 3.125-25 μg/ml for varying times. After washing twice with PBS, the cells were resuspended in Annexin V binding buffer (10 mM HEPES-NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$). After centrifugation, the cells were incubated in 500 μl of the same buffer containing 1.25 μl FITC-conjugated Annexin V (BD PharMingen, San Diego, CA, USA) at room temperature for 15 min in the dark. Data acquisition and analysis used a Becton–Dickinson FACSan flow cytometer (Franklin Lakes, NJ, USA) with CellQuest software (Becton–Dickinson, Oxford, UK).

TUNEL assay

THP-1 cells were cultured in plates and treated with 10 μM taxol or 3.125-25 μg/ml of the *Lcr*S_{5-30} for 24hrs. After PBS wash, 1× 10^6 cells were fixed with 4% formaldehyde in PBS for 10 min at room temperature and permeabilized with 80% ethanol at -20°C. DNA fragments of apoptotic cells were labeled with 57 μL biotin and 3 μL catalyzed by terminal deoxynucleotidyl transferase (TdT) per sample for 2 h. Biotinylated nucleotides were detected using 100 μL streptavidin-horseradish peroxidase (HRP) conjugate. Diaminobenzidine reacted with the labeled sample to generate an insoluble colored substrate at the site of DNA fragmentation. Slides were counterstained with methyl green for morphological evaluation and characterization of normal

and apoptotic cells using light microscopy. A total of 200 cells per sample were analyzed, and the numbers of apoptotic cells were expressed as percentages of the total cells.

Assessment of mitochondrial membrane potential (ΔΨm)

ΔΨm was measured using the lipophilic cation JC-1, which has potential-dependent accumulation in mitochondria indicated by a fluorescence emission shift from green (JC-1 in monomeric form, 527 nm) to red (JC-1 in aggregative form, 590 nm). Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. Purified lymphocytes, monocytes and THP-1 cells were aliquoted to 5×10^5 cells/ml in supplemented RPMI medium and incubated with or without *Lcr*$S_{5\text{-}30}$ for varying time intervals and doses ranging from 3.125 to 25 μg/ml at 37°C in the presence of 5% CO_2. Cells were then washed twice with PBS prior to staining with 1 μM JC-1 in dimethyl sulfoxide (DMSO) (Gibco Invitrogen) for 30 min in the dark at 37°C, and immediately analyzed with a Becton–Dickinson FACSan flow cytometer at 488 nm excitation.

Detection of activated caspase-9

Lymphocytes, monocytes and THP-1 cells (5×10^5 cells/ml) were collected after incubation with *Lcr*$S_{5\text{-}30}$ for varying times and at doses ranging from 3.125 to 25 μg/ml at 37°C in the

presence of 5% CO_2. After incubation, cells were fixed with 4% formaldehyde in PBS for 5 min at room temperature, followed by permeabilization with saponin buffer (0.04% saponin, 50 mM glucose, 0.1% sodium azide) in PBS. Intracellular active caspase-9 subunits were detected by incubation with FITC-conjugated anti-active human caspase-9 antibody (BD Pharmingen) in the presence of rabbit serum (Sigma-Aldrich) to block nonspecific binding. Substrate cleavage of released free FITC fluorescence intensities were recorded with a Becton-Dickinson FACS-Calibur flow cytometer with excitation wavelength at 488 nm and emission wavelength at 520 nm.

Western blot analysis

Monocytes and THP-1 cells were harvested from cultures after treatment with the *Lcr*S_{5-30} for varying time intervals, 3-24 h. Cells were lysed with buffer containing 1% Triton X-100, 50 mM Tris (pH 7.5), 10 mM EDTA, 0.02% NaN_3, and a protease inhibitor mixture (Sigma-Aldrich). After 1 freeze-thaw cycle, cell lysates were centrifuged at 14,000 × *g* for 30 min at 4°C. The supernatants were collected and boiled in the sample buffer for 5 min. After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane (Millipore), blocked overnight at 4°C in PBS-T (PBS plus 0.5% Tween 20) containing 10% skim milk, and probed with Abs against cytochrome *c*, pro-caspase 3, active caspase 3, and β-actin (Calbiochem, San Diego, CA,

USA) for 1h at room temperature. After washes with PBS-T, blots were incubated with a 1/5000 dilution of HRP conjugated goat anti-mouse or anti-rabbit IgG (Calbiochem) for 1 h at room temperature. Protein bands were developed using ECL Plus Western Blotting Detection System (Pierce).

RT-PCR

Total cellular RNA was extracted using an Ultraspec-II RNA isolation system (Biotec, Houston, TX, USA) following the manufacturer's instructions. The concentration of RNA was quantitated by spectrophotometry at 260 nm. cDNA in a total volume of 20 μL was prepared after reverse transcription of cellular RNA (5 μg) with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) using an 20-mer of oligo(dT) as the primer. cDNA (3 μL) was added to PCR buffer containing primers at 1.5 μM each, $MgCl_2$ (1.5 mM), dNTPs (0.2 mM each), and 1 U of *Taq* DNA polymerase (Promega) in a total reaction volume of 50 μL. The oligonucleotide primers for human Bcl-2 (5'-ACAACATCGCCCTGTGGATGA-3' and 5'-ATAGCTGATTCGACGTTTTGCC-3'), Bax (5'-GGAATTCTGACGGCAACTTCAACTG GG-3' and 5'-GGAATTCTTCCAGATGGTGAGCGAGG-3'), and β-actin (5'-AGCGGGAA ATCGTCGTG-3' and 5'-CAGGGTACATGGTGGTGGTGCC-3') were used according to previously published sequences (Lin et al., 2002). Thirty-five cycles were used for Bcl-2 and Bax,

and 30 cycles for β-actin (95°C for 1 min, 55°C for 2 min, and 72°C for 3 min) using a PCR controller (GeneAmp PCR System 2400; PerkinElmer, Wellesley, MA, USA). The PCR products were separated by 1% agarose gel electrophoresis, stained with 0.5μg/ml ethidium bromide, and viewed with UV light.

Cytokine assays

Lymphocytes, monocytes and THP-1 cells, 5×10^5 cells/ml, were stimulated with 1μM LPS and 25μg/ml *Lcr*$S_{5\text{-}30}$. After 24h, culture supernatants were collected, centrifuged and stored at -20°C for cytokines and chemokine analysis. Quantitative human IL-1β, IL-6, IL-8, TNF-α and TGF-β1-specific ELISAs (DuoSet, R & D systems, Minneapolis, MN, USA) were performed in a 96-well plate and detected at 450nm with a microplate reader (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All experiments were performed at least 3 times, each time in triplicate. Data were analyzed by multivariate ANOVA test. If a significant difference was found, a least significant differences (LSD) multiple comparison test was used to identify significant groups. Statistical analyses used The Statistical Software Package for the Social Sciences, version 12.0.1 for Windows (SPSS Inc., Chicago, IL, USA). A P value < 0.05 was considered statistically significant.

RESULTS

Probiotic Lcr preferentially induces apoptosis of a human monocytic leukemia-derived cell line

Following incubation of THP-1 cells with *Lactobacillus* conditioned media, the relative apoptosis promoting activities of probiotics were evaluated by Annexin-V FITC and PI staining in cell-free culture supernatants followed by flow cytometry. The capabilities of *Lactobacillus* strains' supernatants to induce THP-1 cell apoptosis were determining by culturing the cells with 25μg/ml of each supernatant for 24 h. *Lcr*S significantly increased the proportion of Annexin-V positive cells (24.58% ; $P<0.001$), whereas the *L. casei* and *L. rhamnosus* strains induced limited Annexin-V positive staining (2.17% and 4.53%, respectively; Fig. 1).

Partial characterization of the Lcr apoptosis-inducing factor(s)

*Lcr*S was divided into 4 four fractions based on molecular weight using ultrafiltration membranes: <5 kDa, 5-30 kDa, 30-50 kDa and >50 kDa. These fractions induced, respectively, 8.03%, 77.36%, 16.91% and 14.43%Annexin-V positive cells. The *Lcr*$S_{5\text{-}30}$ fraction promoted apoptosis more effectively than the others ($P<0.001$, Fig. 2A).

Then, *Lcr*$S_{5\text{-}30}$ was subjected to various treatments in order to identify the nature of the

compounds responsible for the promotion of apoptosis of THP-1 cells. Heating did not decrease the apoptosis-promoting effect, suggesting that the apoptosis-promoting compounds were heat-stable. Proteinase K and trypsin treatments reduced Annexin-V positive cells by 69.8% and 65.53%, respectively, compared to an untreated fraction (P<0.001). These results suggested that proteinaceous compounds or domain(s) may be involved with promoting apoptosis (Fig. 2B).

Selective apoptosis promotion by LcrS$_{5-30}$ on monocytes, but not intestinal epithelial cells

THP-1 cells were analyzed by TUNEL assay, while the human colonic epithelial carcinoma cell line (HT-29) cells were analyzed by Annexin V-FITC and PI double staining using flow cytometry. Exposure of THP-1 cells to varying concentrations of *Lcr*S$_{5-30}$ (3.125 to 25μg/ml) led to dose-dependent apoptosis induction (Fig. 3A and 3B). In contrast, the majority of HT-29 cells did not undergo apoptosis after exposures to the same concentrations of *Lcr*S$_{5-30}$ (Fig. 3C).

Dose- and time dependence of LcrS$_{5-30}$-induced cytotoxicity

In order to quantify apoptosis induction of immune cells by *Lcr*S$_{5-30}$, both monocytes and lymphocytes were purified from PBMC samples and subjected to similar treatments. Double staining with Annexin-V FITC and PI confirmed time- and dose-dependent apoptosis promotion of lymphocytes, monocytes and THP-1 cells by *Lcr*S$_{5-30}$. To determine the timing of apoptosis

induced by *Lcr*S_{5-30}, cultured cells were treated with 25 μg/ml of *Lcr*S_{5-30} for varying times. Flow cytometry analyses showed that *Lcr*S_{5-30} induced monocytes and THP-1 apoptosis in a time-dependent manner. *Lcr*S_{5-30} also promoted apoptosis of lymphocytes at 12 to 24 h after treatment (Fig. 4A).

To determine the dose of *Lcr*S_{5-30} required to induce apoptosis, cells were treated with different concentrations of *Lcr*S_{5-30} for 24 h. These results showed that 3.125 μg/ml of *Lcr*S_{5-30} effectively induced apoptosis of monocytes and lymphocytes. By comparison, 25μg/ml of *Lcr*S_{5-30} promoted significant apoptosis of THP-1 cells (Fig. 4B).

Apoptosis of monocytes by LcrS$_{5-30}$ via a mitochondrial pathway

The loss of mitochondrial membrane potential (*ΔΨm*) is an important event in apoptosis. The carbocyanine dye JC-1 detects changes in *ΔΨm* due to its dual emission characteristics (Cossarizza et al., 1996). A two-parameter fluorescence display of JC-1-stained monocytes showed that most of the cells emitted relatively lower levels of green fluorescence, while a subpopulation exhibited reduced JC-1 aggregation and an increase in green fluorescence emission, indicating a decrease of *ΔΨm*. Exposure of purified monocytes to 3.125μg/ml and 6.25μg/ml of *Lcr*S_{5-30} for 24 h did not induce significant changes in emission of JC-1 (11.59% and 13.71%,

respectively; % of depolarized $\Delta\Psi m$ cells).However, 12.5μg/ml*Lcr*S_{5-30} strongly induced depolarized $\Delta\Psi m$ cells (56.43%, $P<0.001$, Fig. 5A).

In addition, a high correlation has been previously shown between $\Delta\Psi m$ in isolated mitochondria and fluorescence ratio (mean red fluorescence intensity/mean green fluorescence intensity corresponding to the FL2/FL1 ratio). Thus, to determine the $\Delta\Psi m$ values for lymphocytes, monocytes and THP-1 samples after *Lcr*S_{5-30} treatment, the fluorescence ratios were evaluated (Fig. 5B). The FL2/FL1 ratio was decreased as early as 3 hours after *Lcr*S_{5-30} treatment. For the tested cells, the maximum loss of $\Delta\Psi m$ was reached at approximately 3h, and remained at this level until 12 h after treatment.

To understand the modulation in the apoptotic signaling pathway by *Lcr*S_{5-30}, expressions of the Bcl-2 family proteins Bcl-2 and Bax were analyzed by RT-PCR (Fig. 6A). After treatment with *Lcr*S_{5-30} for monocytes and THP-1 cells, the mRNA levels of Bcl-2 decreased, whereas that of Bax mRNA expression increased in a time-dependent manner (Fig. 6A). The release of cytochrome *c* from mitochondria to cytoplasm was determined by Western blot (Fig. 6B). Also, the cleavages of pro-caspase 9 and pro-caspase 3 into their active forms were detected 3 to 24 h after *Lcr*S_{5-30} treatment (Fig. 6B and 6C). These results indicated that *Lcr*S_{5-30} regulated the

apoptosis of monocytes and THP-1 cells via expressions of mRNAs, including Bcl-2 and Bax, and proteins, including cytochrome *c*, caspase 9 and caspase3, by a mitochondrial pathway.

Inhibition of LPS-induced inflammatory cytokines after treatment with LcrS$_{5-30}$

The apoptosis-inducing effects on pro-inflammatory cells by *Lcr*S$_{5-30}$ implied its potential for regulating the secretions of pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α). Lymphocytes, monocytes and THP-1 cells were treated with *E. coli*-derived LPS and *Lcr*S$_{5-30}$, and the changes of inflammatory cytokines secretions were evaluated by ELISA (Fig. 7). As expected, LPS-treated cells showed elevated levels of inflammatory cytokines. However, cytokines expressions were markedly reduced after simultaneous incubation with *Lcr*S$_{5-30}$ (P<0.01, Fig. 7). These experiments suggested that *Lcr*S$_{5-30}$ was capable of downregulating the secretion of pro-inflammatory cytokines.

LcrS$_{5-30}$ induces TGF-β production and promotes TGF-β-independent apoptosis

TGF-β1 is a key regulatory cytokine involved in anti-inflammation that counteracts IL-1 and TNF-α and modulates cellular functions, such as homing, cellular adhesion, chemotaxis and T-cell homeostatic regulation (Luethviksson and Gunnlaugsdottir, 2003).In contrast to the pro-inflammatory cytokines described above, dramatic increases of TGF-β1 levels were observed

following treatment with *Lcr*S_{5-30} (Fig. 8A).

TGF-β regulates a wide array of biological functions, including apoptosis (Heldin et al., 1997). TGF-β production was increased by *Lcr*S_{5-30} treated THP-1 cells. It was reasonable to postulate that apoptosis might be triggered by TGF-β rather than *Lcr*S_{5-30}. To confirm this assumption, a pharmacological inhibitor, SB431542, was applied. The percentages of apoptotic cells in 10 ng/ml and 1 ng/ml TGF-β exposed preparations were 18.9 ± 0.44% and 16.57 ± 0.67%, respectively (Fig. 8B). Treatments with 12.5 μg/ml and 25μg/ml of *Lcr*S_{5-30} induced dissimilar levels of apoptosis of THP-1 cells, which were 26.35 ± 0.77% and 74.96 ± 0.38%, respectively. One ng/ml TGF-β and 12.5 μg/ml *Lcr*S_{5-30} induced apoptosis were SB431542 sensitive. Nevertheless, 25μg/ml of *Lcr*S_{5-30} induced dramatic changes in the levels of apoptosis of THP-1 cells, and SB431542 could not reverse this phenomenon (Fig. 8B). Collectively, these results indicated that *Lcr*S_{5-30} was capable of inducing TGF-β-independent cell death.

DISCUSSION

In the present study, several *Lactobacillus* strains were investigated for their differential capabilities to promote apoptosis of THP-1 cells. *Lcr* exhibited more potent apoptosis inducing capability than the *L. casei* and the *L. rhamnosus* strains. Probiotics, as preventative or therapeutic agents against IBD, are an attractive, alternative approach for the attenuation of mucosal inflammation. Many clinical studies during recent decades demonstrated that probiotic species possessed beneficial effects for IBD (Miele et al., 2009; Bibiloni et al., 2005; Kruis et al., 2004). However, inconsistency among some results might have resulted from differences in probiotic species or strains. These inconsistencies have drawn attention to understanding the mechanisms of probiosis by specific strains for possible clinical applications.

Initial characterization of the apoptosis inducing factor(s) in *Lcr*S employed MWCO ultrafiltration. The apoptosis inducing factor(s) were present in the 5-30 kDa fraction. Several studies have described apoptosis promotion via factor(s) produced by probiotics, such as for human breast cancer cells by fermented soy milk (Chang et al., 2002), human γδ T cells by *E. coli* Nissle 1917 supernatant (Guzy et al., 2008) and for human myeloid leukemia-derived cells by *L. reuteri* (Iyer et al., 2008). The identities of these factor(s) have not been determined. The

Lcr-derived apoptosis-inducing factor(s) are small heat stable proteins or peptides components, based on results of heat inactivation and protease treatment experiments.

Pathologically, IBD is characterized by a high density of mucosal cells within the inflamed tissues that mainly consist of activated T cells, peripheral blood neutrophils and monocytes/macrophages (Lügering et al., 2006). Extensive studies in recent years have shown that activation and increased survival time of leucocytes might contribute to the severity of intestinal inflammation and clinical relapses in both CD and UC (Itoh et al., 2001). Activation-induced cell death (AICD) is an important mechanism to limit the number of active monocytes and lymphocytes, and to terminate an immune response. *Lcr*$S_{5\text{-}30}$ was effective for promoting lymphocyte/ monocyte/ THP-1 cell apoptosis in both dose- and time-dependent manners.

Despite a paucity of data regarding probiotics for promoting apoptosis of immune effector cells as an alternative IBD therapy, experiments with γδ T cell going through programmed cell death by *E. coli* Nissle and human myeloid leukemia-derived cells proceeding through TNF-induced apoptosis by *L. reuteri* provided a rational basis for an apoptosis-inducing strategy for IBD therapy. Our study with monocytes showed that 12.5 μg/ml and 25 μg/ml of *Lcr*$S_{5\text{-}30}$ could disrupt the mitochondrial membrane potential, and apoptotic cell death was induced within 3 hrs

following exposure to 25 μg/ml of *Lcr*S_{5-30}. Moreover, the increase of the Bax/Bcl-2 ratio, release of cytochrome *c*, and activation of caspase-9 and caspase-3, showed that *Lcr*S_{5-30} is capable of inducing monocytes apoptosis via a mitochondrial pathway.

An anti-TNF antibody, infliximab, is an FDA-approved treatment for CD and UC. Several studies demonstrated that infliximab induced monocytes apoptosis (Lügering et al., 2001) and a loss of CD68+ monocytes, as well as CD4+ and CD8+ T lymphocytes, in the lamina propria (Baert et al., 1999). Also, infliximab exerted killing activity on human peripheral blood T cells by as much as 50.6% after 18 h culture with 5 μg/mlinfliximab (Sabatino et al., 2004). In our study, *Lcr*S_{5-30} promoted apoptosis of lymphocytes, monocytes and THP-1 cells by as much as 93%, 97% and 74%, respectively. The induction of apoptosis did not require the Fas/FasL signal transduction pathway. Rather, it involved upregulation of Bax/Bak followed by mitochondrial release of cytochrome *c* (Sabatino et al., 2004). Interestingly, the extents of immune effector cells apoptosis induced by infliximab and probiotic *Lcr* were similar. Also, given the safety history of probiotics, *Lcr* could be a useful adjunctive treatment for IBD patients.

The loss of intestinal epithelial cell (IEC) function, and subsequent changes in epithelial tight junction protein expressions and IEC apoptosis are also critical components for the initiation

and perpetuation of IBD (Xavier and Podolsky, 2007). Probiotics have been investigated for protective effects by regulating IEC survival for treating and preventing intestinal inflammation. *In vitro* results indicated that LGG reduced intestinal epithelial apoptosis by upregulating the expressions of anti-apoptotic and cytoprotective genes (Lin et al., 2008). Also, soluble factors from LGG stimulated anti-apoptotic Akt activation and prevented cytokine-mediated apoptosis (Yan et al., 2007). In this study, *Lcr*$S_{5\text{-}30}$ was quite unique in that it promoted apoptosis of monocyte-like cells, but not of IEC. These results are consistent with previous studies in which probiotics could protect intestinal integrity by promoting survival of IEC.

Lcr$S_{5\text{-}30}$ possessed potent inhibitor(s) for LPS induced pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α, and chemokine, IL-8. The suppression of IL-1β, IL-6, TNF-α and IL-8 suggested that *Lcr*$S_{5\text{-}30}$ might concomitantly suppress lymphocyte/monocyte/THP-1 chemotaxis and cellular activation. TNF-α is a crucial proinflammatory cytokine in various inflammatory disorders. Blocking of TNF-α is efficient for the treatment of patients with CD and UC (Rutgeerts et al., 2004). Interestingly, studies have indicated that apoptosis was indeed linked to the clinical efficacies of various anti-TNF agents. TNF-α inhibitors, such as infliximab and adalimumab, have shown clinical efficacy for IBD and exerted strong apoptotic effects (Baert et al., 1999). However, another anti-TNF agent, etancercept, did not establish a positive influence on the

course of CD (Van den Brande et al., 2003) due to a failed apoptosis inducing ability in monocytes and lymphocytes. Our results indicated that *Lcr*S_{5-30} possessed both TNF-α suppression and apoptosis promotion capabilities, as seen with infliximab.

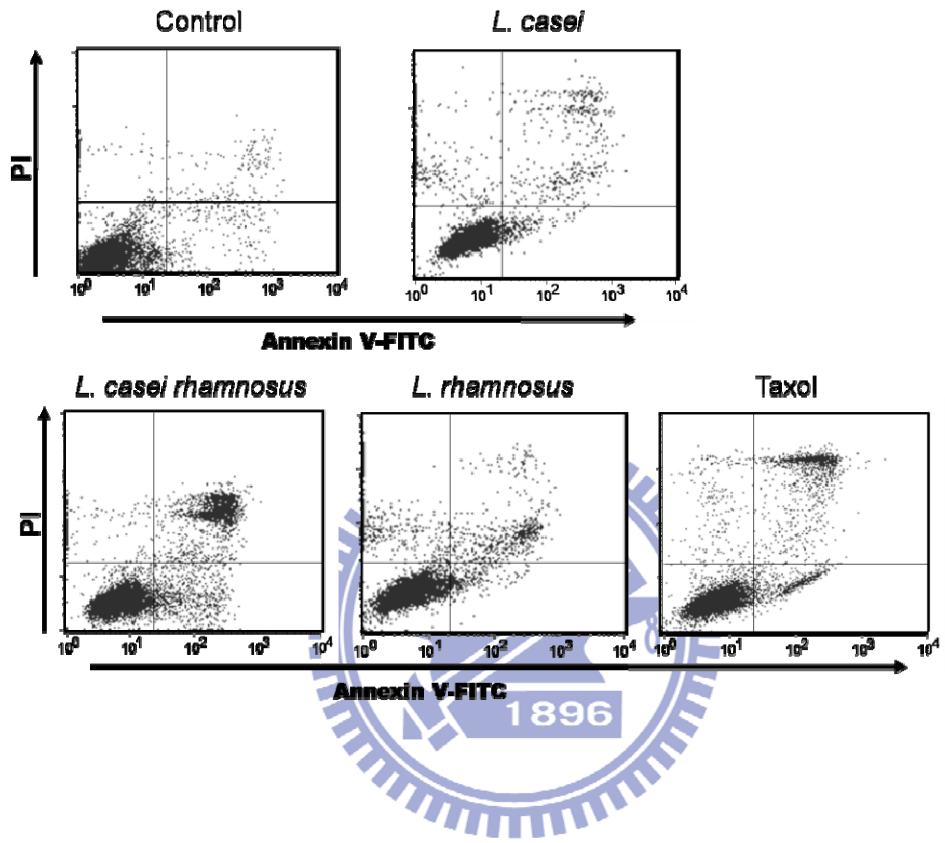
TGF-β is a potent anti-inflammatory cytokine and has a vital role for suppressing the activation and proliferation of inflammatory cells (Letterio and Roberts, 1998). Also, TGF-β has been implicated for an essential role in disease remission by promoting the maturation of intestinal epithelial cells and for healing wounds and ulcers (Kader et al., 2005). *L. paracasei* has been reported to induce populations of regulatory CD4+ T cells, which produce high levels of modulatory cytokines, IL-10 and TGF-β (von der Weid et al., 2001). *Lactobacilli* modulate cytokine production in bone-marrow-derived dendritic cells with a net effect of altering overall cytokine profiles in a species-dependent manner (Christensen et al., 2002). One study also showed that pediatric IBD patients in remission compared to those with active disease had higher levels of TGF-β1 (Kader et al., 2005). In this study, because *Lcr*S_{5-30} could induce high TGF-β1 production, but not IL-10 (data not shown), by lymphocytes, monocytes, and THP-1 cells implied that *Lcr*S_{5-30} altered the balance between pro-inflammatory and anti-inflammatory cytokines, and highlights their important immunomodulatory roles in inflammatory diseases.

TGF-β can trigger apoptosis in myeloid leukemia cells (Heldin et al., 1997). SB-431542, a specific inhibitor of TβRI, inhibits the TGF-β-induced apoptosis in several cell types by blocking TGF-β signaling. Upregulated TGF-β production promotes monocytes apoptosis and contributes to the prevention of tissue injury. It was important to confirm the consequences of TGF-β production in regulating the apoptosis of monocytes following *Lcr*S_{5-30} treatment. SB-431542 significantly blocked TGF-β1- and 12.5μg/ml - induced apoptosis. The higher dose of *Lcr*S_{5-30} induced a significant amount of apoptosis in THP-1 cells, but this was inefficiently abrogated by SB 431542. Thus, *Lcr*S_{5-30} could trigger TGF-β-independent apoptosis.

In conclusion, probiotic *Lcr* produces heat-stable molecules with a MW range of 5-30 kDa, primarily proteins, which promoted lymphocyte, monocyte and THP-1 cell apoptosis without affecting intestinal epithelial cells. *Lcr*S_{5-30} triggered apoptosis of immune cell *in vitro* by a mitochondrial pathway, but not via the TGF-β signaling pathway. *Lcr*S_{5-30} also inhibited LPS-induced inflammatory cytokines in activated immune cells. This investigation demonstrated a role for *Lcr*S_{5-30} in promoting apoptosis of immune cells and suggests the possibility of a probiotics-based regimen for prevention of IBD.

FIGURES AND LEGENDS

A.



B.

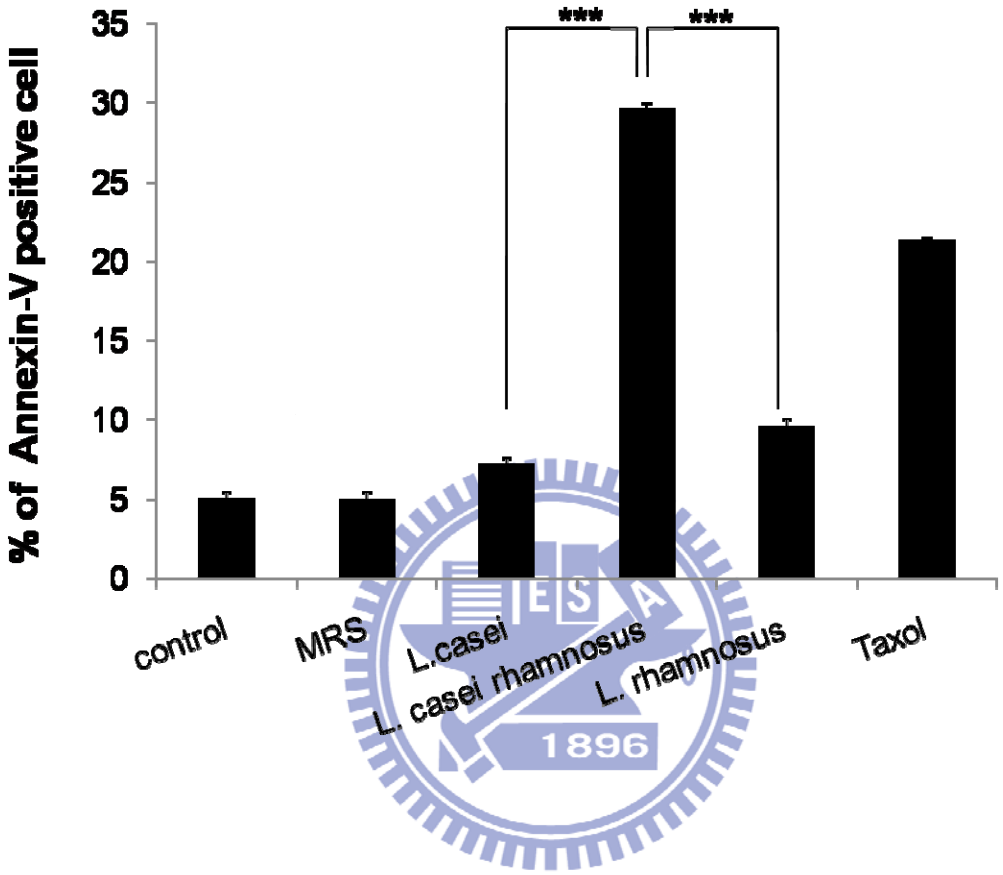
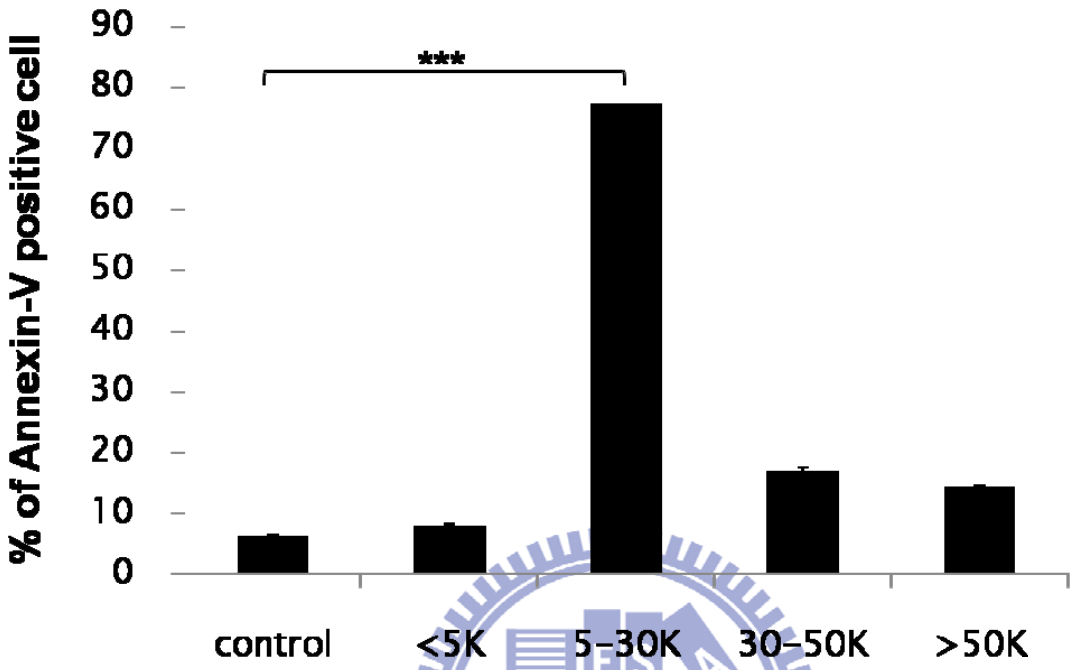

% of Annexin-V positive cell
35
30
25
20
15
10
5
0
***
***
control
MRS
L.casei
L. casei rhamnosus
L. rhamnosus
Taxol

Figure 1. Strain-dependent promotion of apoptosis of THP-1 cells in the absence or presence of probiotic-derived secreted factors.

*Lcr*S significantly increased the numbers of Annexin V positive cells, but fewer were found for *L. casei* and *L. rhamnosus*. Apoptotic THP-1 cells following exposure to 25μg/ml probiotic supernatants were determined by double staining with Annexin V-FITC and Propidium iodide (PI). Cells that were Annexin V positive and PI negative were early apoptosis cells. Cells positive both for Annexin V and PI represented cells in late apoptosis. Results representative of typical donors (panel A) and mean ± standard error of the mean (panel B) for 3 different experiments. Control = complete RPMI cell culture medium. MRS=bacterial culture medium. 1 μM Taxol was a positive control. ***$P < 0.001$ for change versus *Lcr*S.

A.


% of Annexin-V positive cell
90
80
70
60
50
40
30
20
10
0
***
control
<5K
5-30K
30-50K
>50K

B.

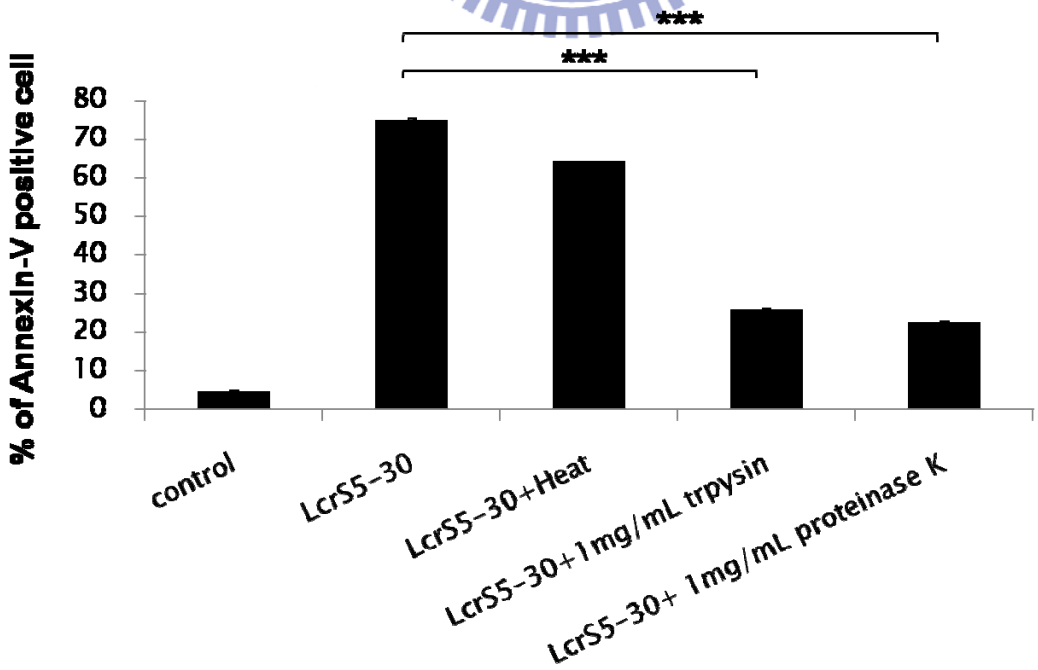
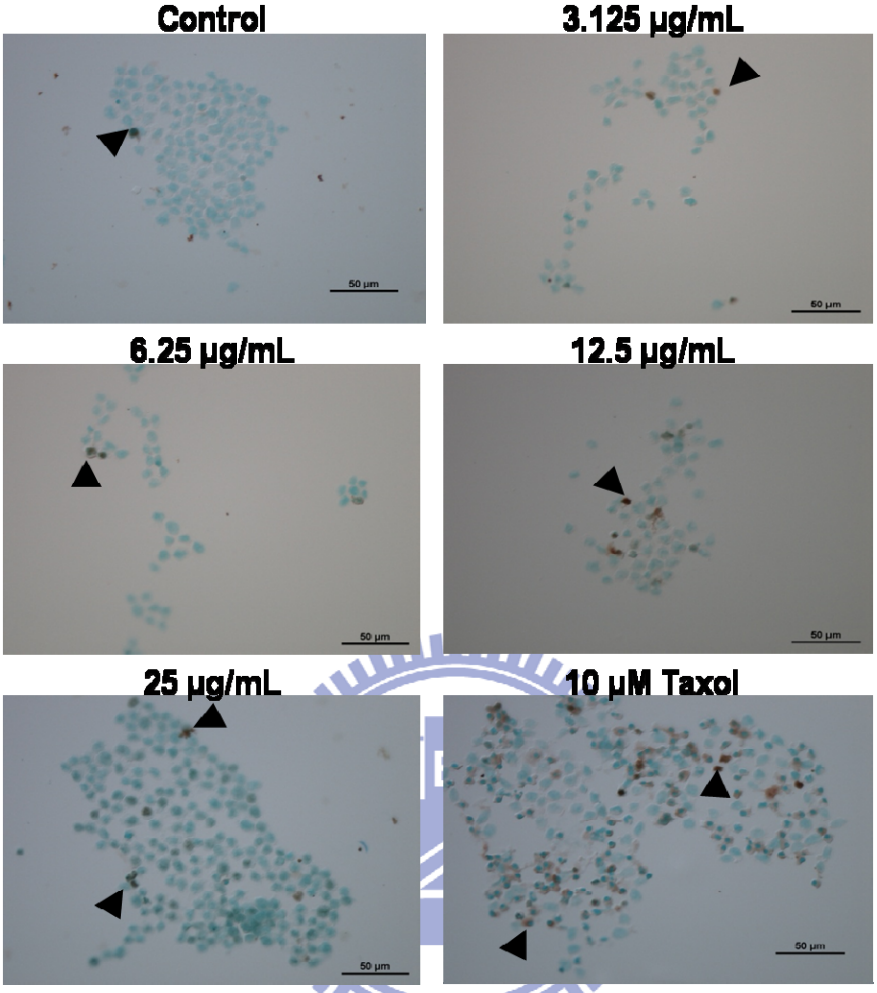
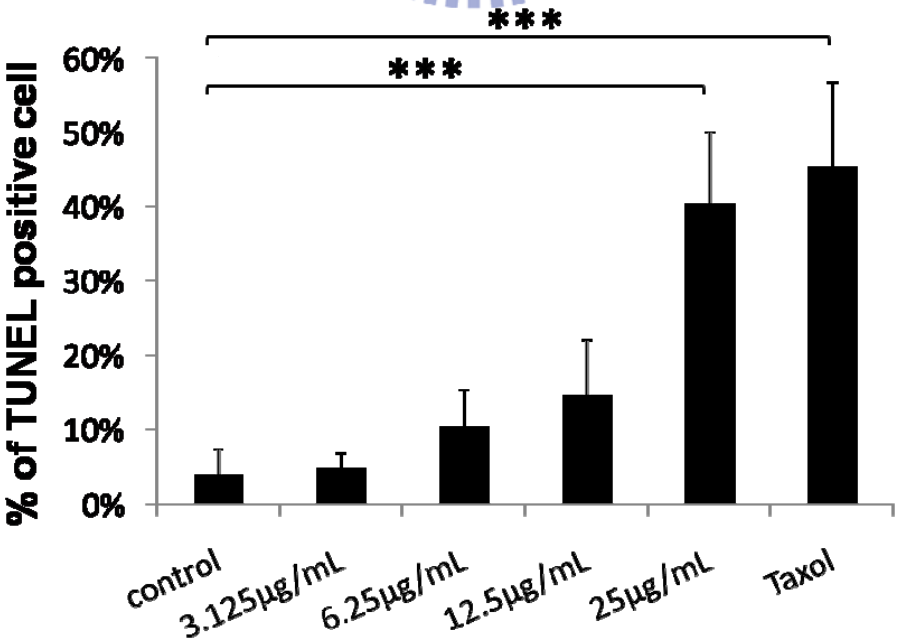

% of Annexin-V positive cell
80
70
60
50
40
30
20
10
0
***
***
control
LcrS5-30
LcrS5-30+Heat
LcrS5-30+1mg/mL trpysin
LcrS5-30+ 1mg/mL proteinase K

Figure 2. Partial characterization of factors promoting THP-1 apoptosis. THP-1 cells were stimulated with 25μg/ml of 4 different fractions (panel A). THP-1 cells were stimulated with *Lcr*S_{5-30} that had either been boiled for 30 mins or treated with 1mg/ml trypsin or proteinase K. After 24 h stimulation, apoptotic THP-1 cells following exposure to 25μg/ml *Lcr*S_{5-30} were determined by double staining with Annexin- V FITC and PI (panel B). Each bar represents mean ± standard error of the mean of 3 individual experiments. ***P< 0.001 for change versus control.

A.


Control
3.125 μg/mL
6.25 μg/mL
12.5 μg/mL
25 μg/mL
10 μM Taxol
50 μm

B.


***
***
% of TUNEL positive cell
60%
50%
40%
30%
20%
10%
0%
control
3.125μg/mL
6.25μg/mL
12.5μg/mL
25μg/mL
Taxol

C.

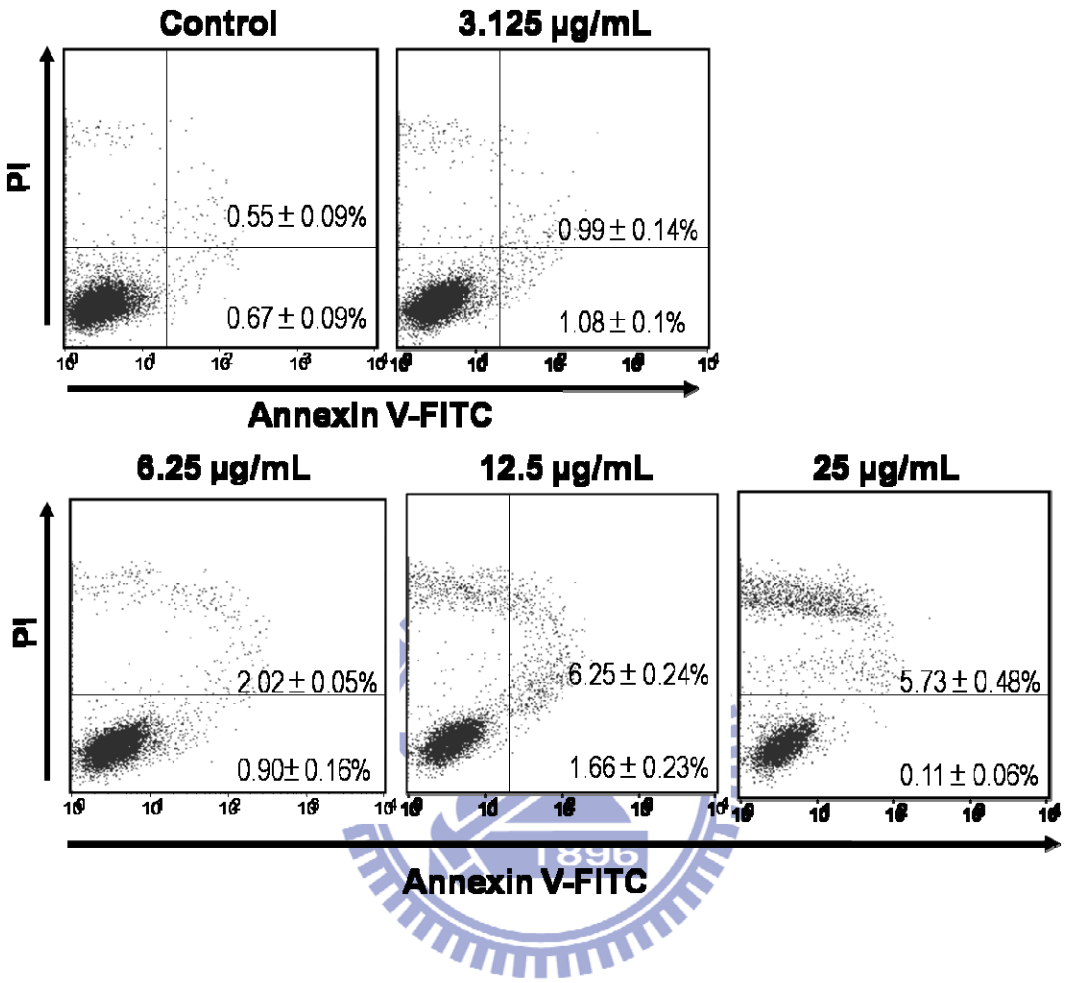
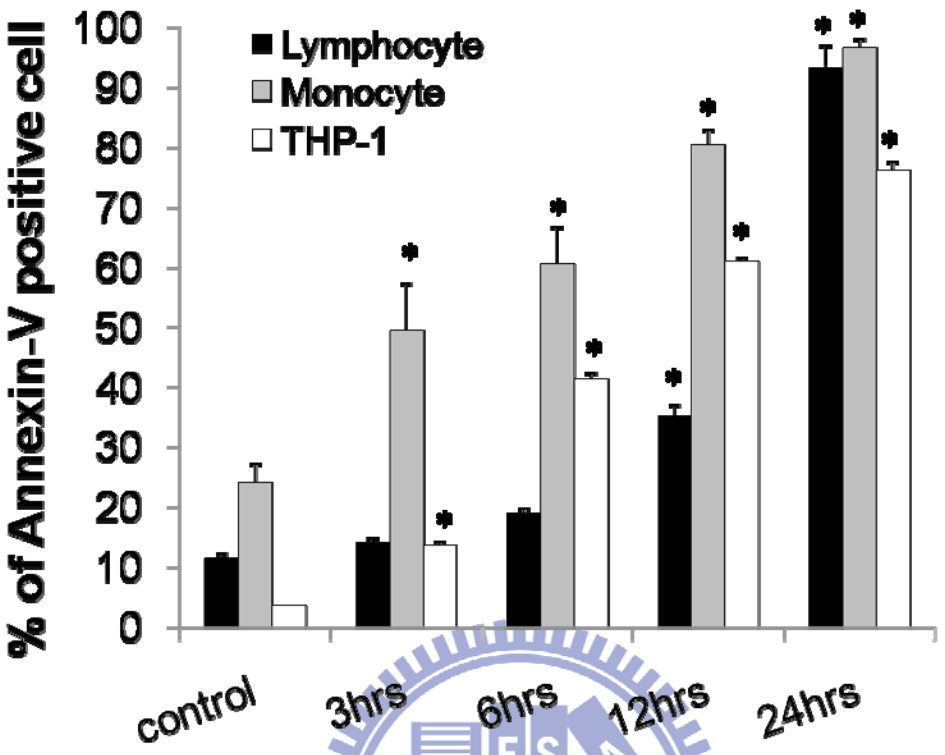

Control
3.125 μg/mL
PI
0.55 ± 0.09%
0.67 ± 0.09%
0.99 ± 0.14%
1.08 ± 0.1%
Annexin V-FITC
6.25 μg/mL
12.5 μg/mL
25 μg/mL
PI
2.02 ± 0.05%
0.90 ± 0.16%
6.25 ± 0.24%
1.66 ± 0.23%
5.73 ± 0.48%
0.11 ± 0.06%
Annexin V-FITC

Figure 3. *Lcr*S_{5-30} promoted apoptosis of monocytes, but not intestinal epithelial cells. THP-1 cells (panels A and B) and HT-29 cells (panel C) were treated with varying concentrations of *Lcr*S_{5-30} or 10 μM Taxol as a positive control for 24h. TUNEL staining was observed by light microscope. Arrows indicate representative apoptotic cells (panel A). The percentage of cells that underwent apoptosis from a representative experiment (panel B). Apoptotic HT-29 cells exposed to *Lcr*S_{5-30} were determined by Annexin V-FITC and PI double staining followed by flow cytometric analysis (panel C). All experiments were performed on at least 3 separate occasions. ***$P < 0.001$ for change versus control.

A.


% of Annexin-V positive cell
100
90
80
70
60
50
40
30
20
10
0
Lymphocyte
Monocyte
THP-1
control
3hrs
6hrs
12hrs
24hrs

B.

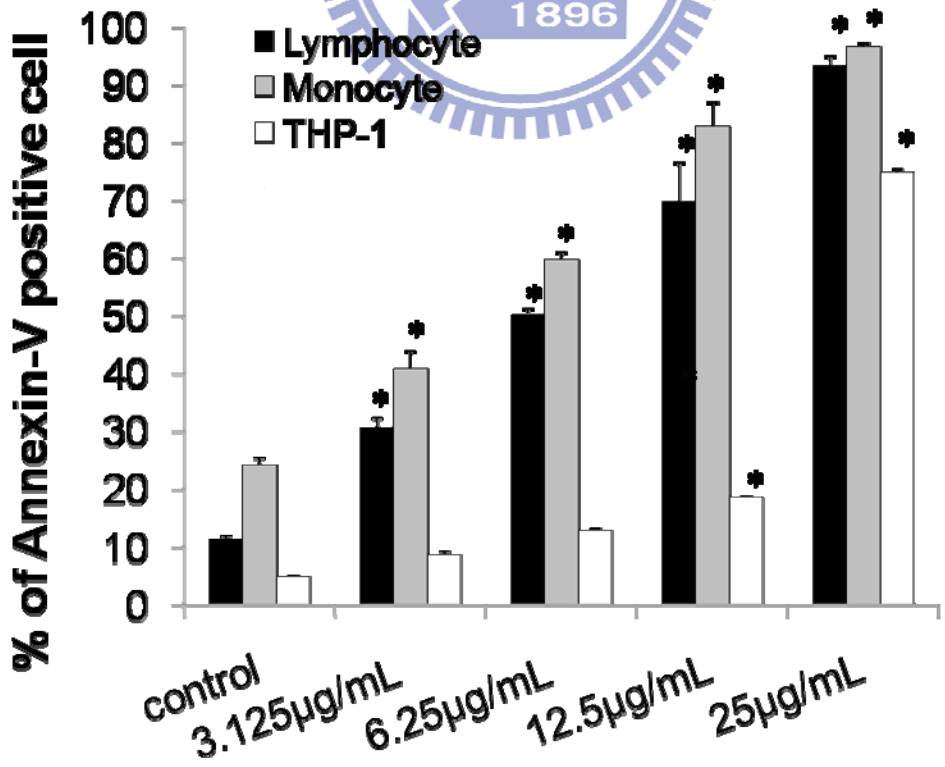
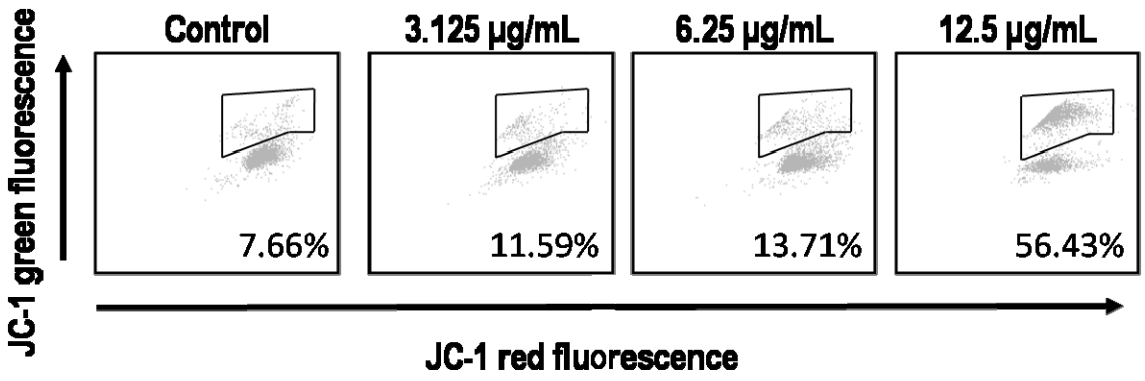

% of Annexin-V positive cell
100
90
80
70
60
50
40
30
20
10
0
Lymphocyte
Monocyte
THP-1
control
3.125μg/mL
6.25μg/mL
12.5μg/mL
25μg/mL

Figure 4. Incubation with *Lcr*$S_{5\text{-}30}$ increased the numbers of Annexin V positive cells in a time- and dose-dependent manner. Lymphocytes (■), monocytes (■) or THP-1 cells (□) (1×10^6 per test) were collected after incubation with *Lcr*$S_{5\text{-}30}$ for varying times (panel A) and for doses ranging from 3.125 to 25 μg/ml (panel B). Cell apoptosis was determined by double staining with Annexin V-FITC and PI. Results are mean ± standard error of the mean from triplicate cultures. **P*< 0.01 for change versus control.

A.


Control
3.125 μg/mL
6.25 μg/mL
12.5 μg/mL
7.66%
11.59%
13.71%
56.43%
JC-1 green fluorescence
JC-1 red fluorescence

B.

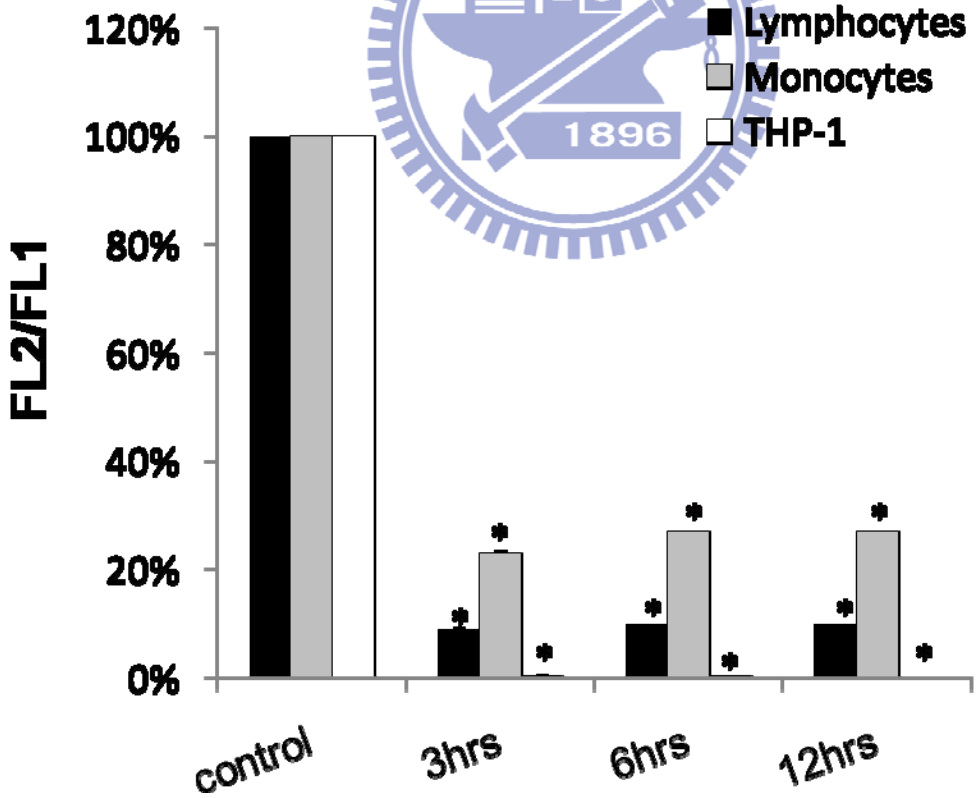
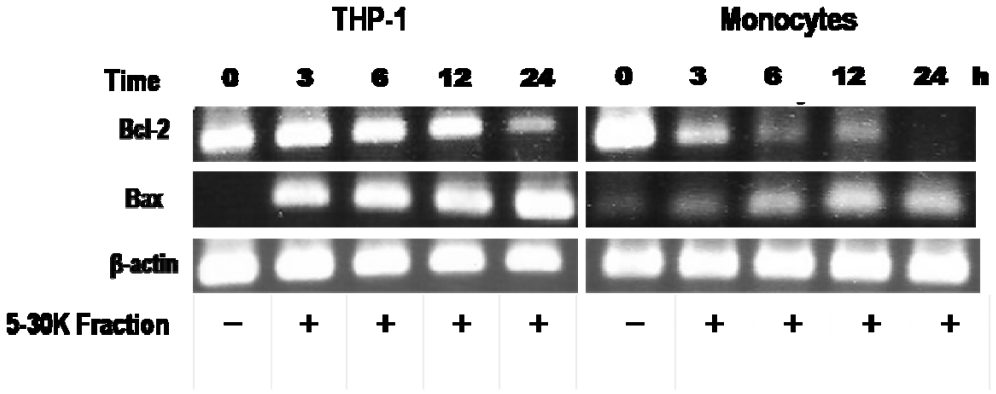
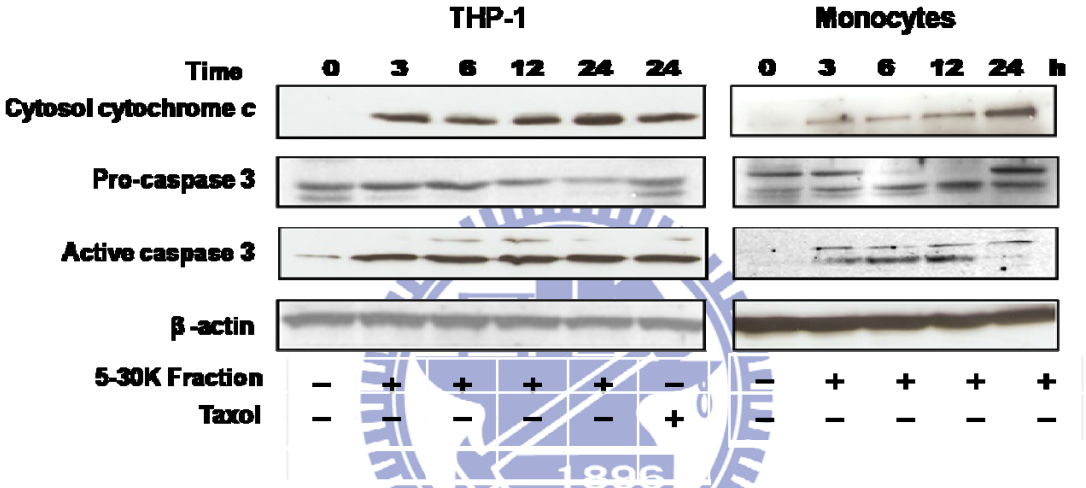

Lymphocytes
Monocytes
THP-1
FL2/FL1
120%
100%
80%
60%
40%
20%
0%
control
3hrs
6hrs
12hrs

Figure 5. Cytofluorometric analysis of mitochondrial membrane potential *(ΔΨm)*. One representative analysis of *ΔΨm* in monocytes after stimulation with varying concentrations of *Lcr*$S_{5\text{-}30}$ (panel A). Respective percentages of cells with depolarized mitochondria (%*ΔΨm*) are indicated in the upper box of each group (7.66, 11.59, 13.71 and 56.43%, respectively). Thus, a decrease in *ΔΨm* corresponds to an increase in percent of monocytes %*ΔΨm*. JC-1 red fluorescence/JC-1 green fluorescence ratio for lymphocytes, monocytes and THP-1 with depolarized *ΔΨm*, with or without the *Lcr*$S_{5\text{-}30,}$ for varying times (panel B). **P*< 0.01 for change versus control.

A.


THP-1
Monocytes
Time
0 3 6 12 24 0 3 6 12 24 h
Bcl-2
Bax
β-actin
5-30K Fraction
– + + + + – + + + +

B.


THP-1
Monocytes
Time
0 3 6 12 24 24 0 3 6 12 24 h
Cytosol cytochrome c
Pro-caspase 3
Active caspase 3
β -actin
5-30K Fraction
Taxol
– + + + + – – + + + +
– – – – – + – – – – –

C.

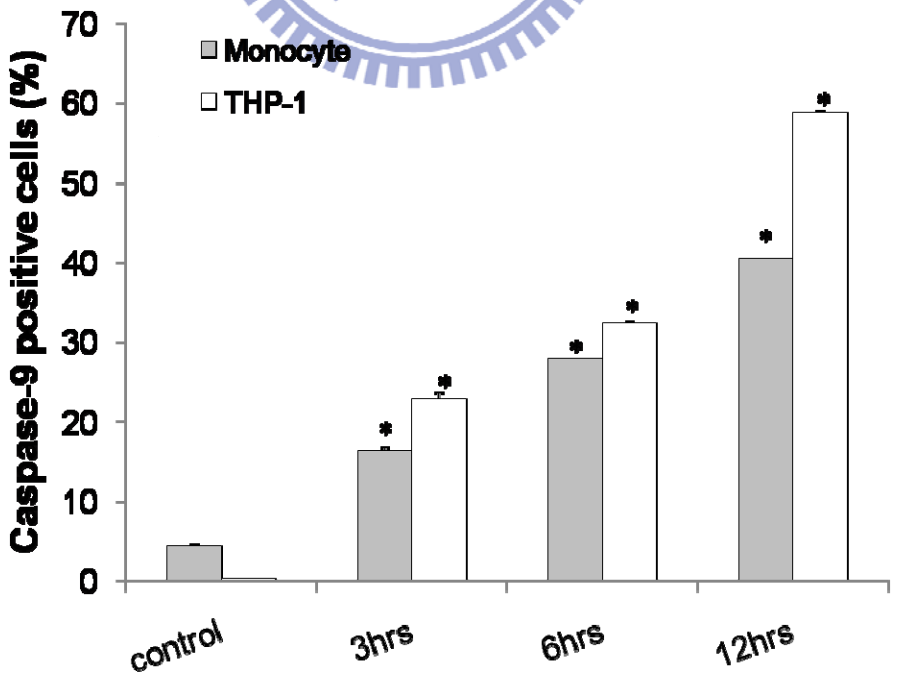
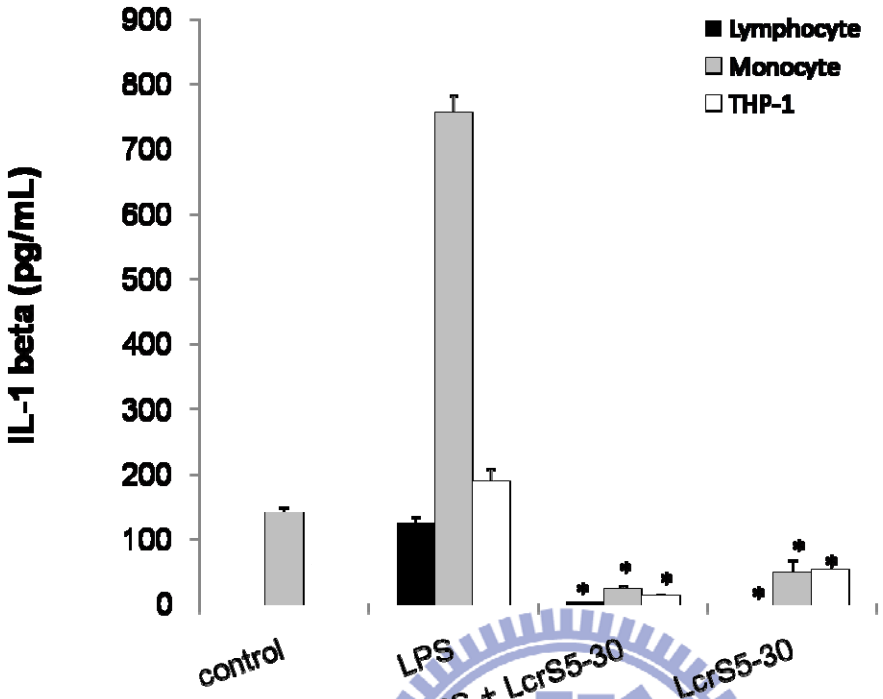
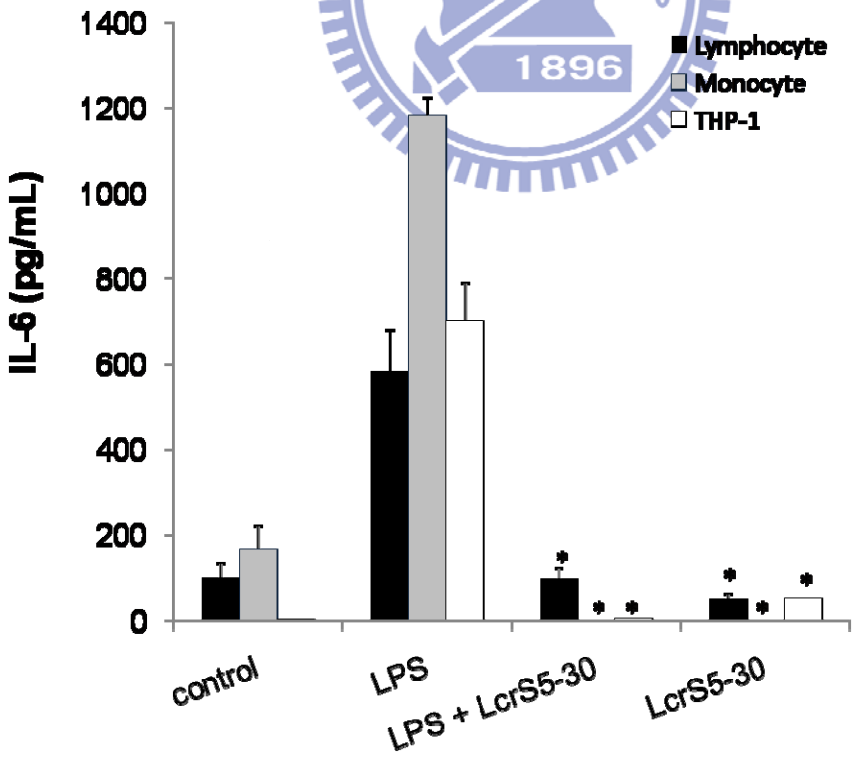

Caspase-9 positive cells (%)
70
60
50
40
30
20
10
0
Monocyte
THP-1
control
3hrs
6hrs
12hrs

Figure 6. *Lcr*S_{5-30} causes decreased Bcl-2 expression, increased Bax and caspase 9 expressions, cytochrome *c* (Cyt c) release and caspase 3 activation. Monocytes and THP-1 cells (1×10^6) were treated with or without 20μg/ml of *Lcr*S_{5-30} for indicated times. mRNA was detected by RT-PCR (panel A), and the protein levels were determined by Western blot (panel B). Both the mRNA and protein expressions of actin were internal controls. Cells were incubated with FITC-conjugated anti-active caspase-9 antibody and analysis by flow cytometry (panel C). Monocytes and THP-1 cells exhibiting positive intracellular active caspase-9 fluorescence were enumerated, and the results are expressed as a percentage of the total number of cells analyzed. Results are mean ± standard error of the mean of 3 individual experiments. **P*< 0.01 for change versus control.

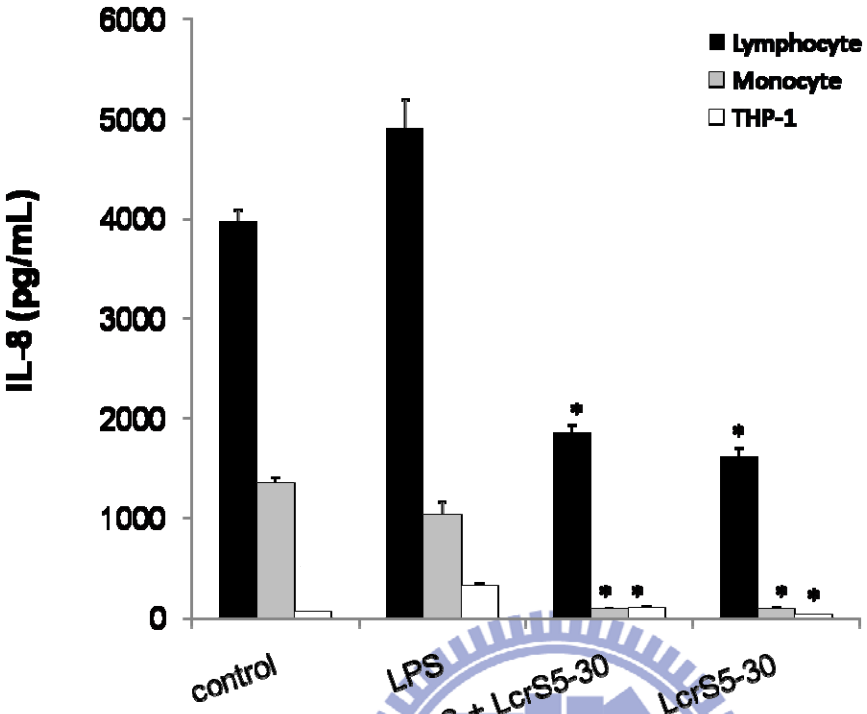
A.


IL-1 beta (pg/mL)
900
800
700
600
500
400
300
200
100
0
Lymphocyte
Monocyte
THP-1
control
LPS
LPS + LcrS5-30
LcrS5-30

B.


IL-6 (pg/mL)
1400
1200
1000
800
600
400
200
0
Lymphocyte
Monocyte
THP-1
control
LPS
LPS + LcrS5-30
LcrS5-30

C.


IL-8 (pg/mL)
6000
5000
4000
3000
2000
1000
0
Lymphocyte
Monocyte
THP-1
control
LPS
LPS + LcrS5-30
LcrS5-30

D.

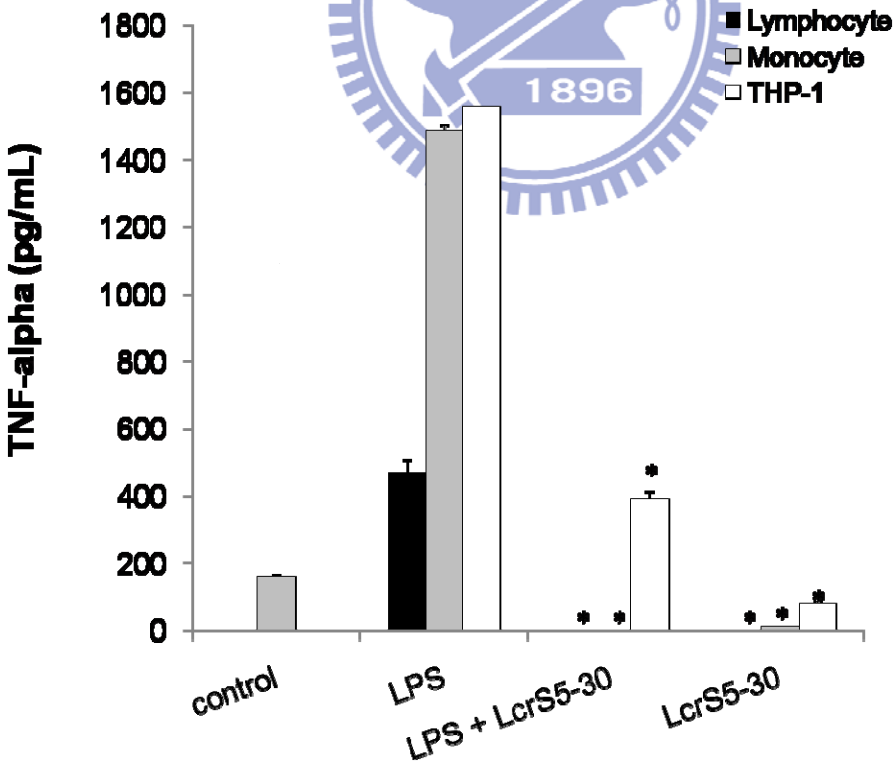
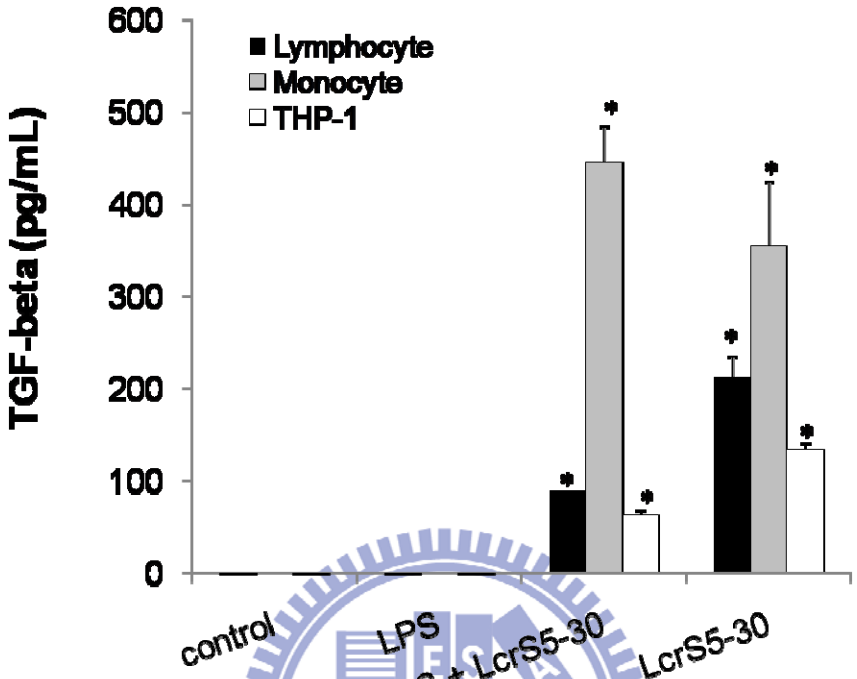

TNF-alpha (pg/mL)
1800
1600
1400
1200
1000
800
600
400
200
0
Lymphocyte
Monocyte
THP-1
control
LPS
LPS + LcrS5-30
LcrS5-30

Figure 7. Inhibition of cytokine production by *Lcr*S_{5-30} by lymphocytes, monocytes and THP-1 cells. Cell culture bioassay were performed by stimulating lymphocytes (■), monocytes (■) or THP-1 cells (□) with *E.coli*-derived LPS and 25 μg/ml of *Lcr*S_{5-30}. Human IL-1β (panel A), IL-6 (panel B), IL-8 (panel C) and TNF-α (panel D) cytokine were determined in culture supernatants by sandwich ELISA following cell culture. Each bar represents mean ± standard error of the mean of 3 individual experiments. **P*< 0.01 for change versus LPS.

A.


600
500
400
300
200
100
0
TGF-beta (pg/mL)
Lymphocyte
Monocyte
THP-1
control
LPS
LPS + LcrS5-30
LcrS5-30

B.

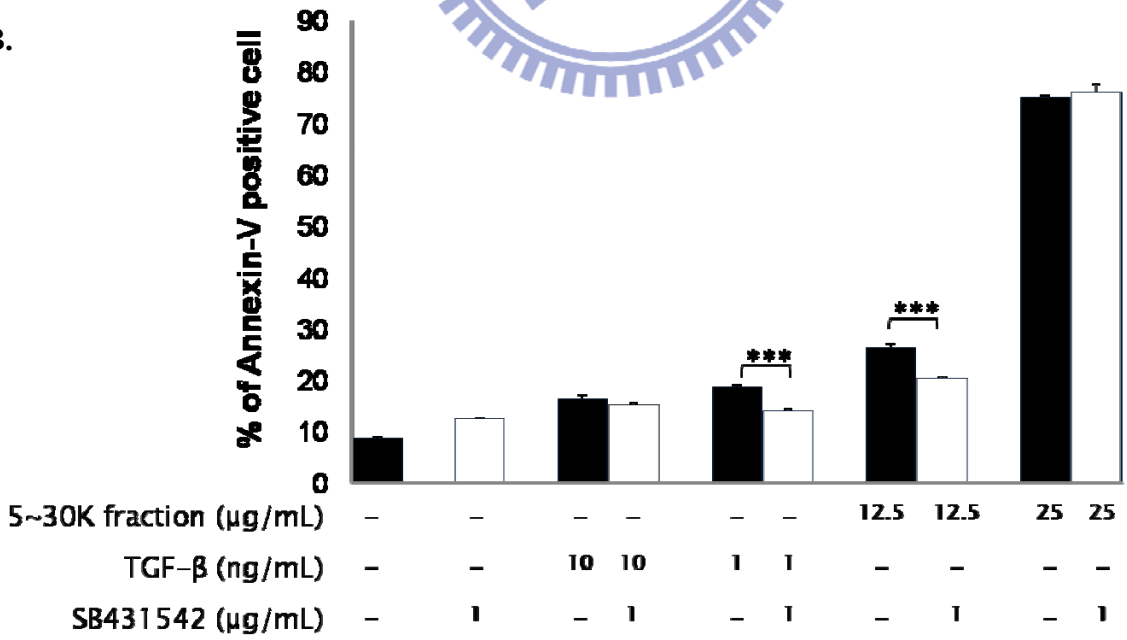

90
80
70
60
50
40
30
20
10
0
% of Annexin-V positive cell
***
***
5~30K fraction (μg/mL) – – – – – – 12.5 12.5 25 25
TGF-β (ng/mL) – – 10 10 1 1 – – – –
SB431542 (μg/mL) – 1 – 1 – 1 – 1 – 1

Figure 8. *Lcr*S_{5-30} induced TGF-β1 production and promoted TGF-β independent apoptosis. Human TGF-β1 quantities were determined by TGF-β1 specific ELISA in culture supernatants following lymphocytes (■), monocytes (■) or THP-1 cells (□) culture (panel A). THP-1 cells were treated with TGF-β1 or *Lcr*S_{5-30} in the absence or presence of 1μg/ml of TGF-β inhibitor, SB431542, for 24 h (panel B). Cell apoptosis was determined by double staining with Annexin V-FITC and PI. T Results are mean ± standard error of the mean from triplicate cultures. **P*< 0.01 for change versus LPS. *** *P*< 0.001 for change versus 1ng/ml SB431542 versus the respective control.

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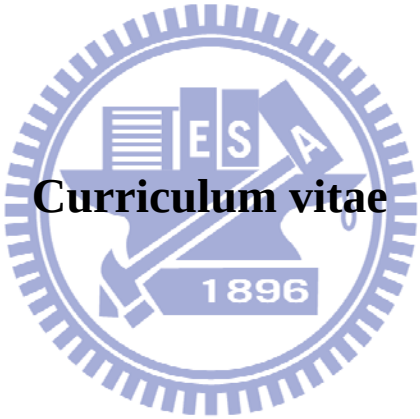

Curriculum vitae

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EDUCATION

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PUBLICATIONS

1. **Chiu YH**, Hsieh YJ, Chang M, Liao KW, Peng KC. (2009). Preferential promotion of apoptosis of monocytes by *Lactobacillus casei rhamnosus* soluble factors. *Clinical nutrition (accepted).* **(IF = 3.203, Subject categories: 12 / 59)**

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