Anti-inflammatory activity of probiotic *Lactobacillus paracasei* in colitis and hepatitis model

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2020

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Anti-inflammatory activity of probiotic *Lactobacillus paracasei* in colitis and hepatitis model

BOONYARUT LADDA

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THE DISSERTATION TITLED

ANTI-INFLAMMATORY ACTIVITY OF PROBIOTIC *LACTOBACILLUS PARACASEI* IN COLITIS AND HEPATITIS MODEL

BY

BOONYARUT LADDA

HAS BEEN APPROVED BY THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT
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IN BIOMEDICAL SCIENCES AT SRINAKHARINWIROT UNIVERSITY

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Probiotics are beneficial microbiota which promote the health of the host in terms of immunomodulation. In this study, four antigens of *Lactobacillus* strains were prepared for the detection of tumor necrosis factor α (TNF-α) secretion in the Hepatocellular carcinoma cell line (HepG-2 cells) and colorectal adenocarcinoma cell (Caco-2 cells). It was found that viable cells, heat-killed cells of *L. paracasei* MSMC39-1, *L. casei* MSMC39-3 and sonicated cell of *Weissella confusa* MSMC57-1 were strong TNF-α secretion inhibitors in HepG-2 cells and Caco-2 cells. The viable cells of *L. paracasei* MSMC39-1 were selected in rats with alcoholic hepatitis and colitis rat models. The levels of the aspartate aminotransferase serum were significantly reduced in hepatitis and colitis rats treated with *L. paracasei* MSMC39-1. TNF-α secretion in liver tissues of hepatitis rats and colon tissue of colitis rats in probiotic *L. paracasei* MSMC39-1 treatment were significantly suppressed. The liver histopathology examination of alcohol-induced hepatitis and dextran sulfate sodium-induced colitis rats indicated fat accumulation in the hepatocytes, and loss of hepatic architecture. Interestingly, liver damage in hepatitis rats was improved by probiotic *L. paracasei* MSMC39-1. In addition, *L. paracasei* MSMC39-1 alleviated colon inflammation in hepatitis rats and colitis rats. Administration of *L. paracasei* MSMC39-1 modulated gut microbiota by increasing the number of genus *Lactobacillus* in alcohol-induced hepatitis rats and dextran sulfate sodium-induced colitis rats. *L. paracasei* MSMC39-1 had potent anti-inflammatory effects in HepG-2 cell line, Caco-2 cell line, improves hepatitis, colitis and modulates gut microbiota. Thus *L. paracasei* MSMC39-1 is able to protect alcoholic hepatitis and colitis. It is also an alternative treatment for other inflammatory diseases and applied in food supplementation products.

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BOONYARUT LADDA

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CHAPTER 1
INTRODUCTION

Lactobacillus is a member of lactic acid bacteria, which is generally used as probiotics in dairy products, food and vegetables, is a rod-shaped, non-spore-forming gram positive, non-spore-forming and catalase negative bacteria. The members of bacteria used as probiotic in this genus are *L. paracasei*, *L. acidophilus*, *L. casei*, *L. plantarum*, *L. fermentum* and *L. reuteri*, *L. brevis* [1]. Moreover, other lactic acid bacteria including Bifidobacteria and *Enterococcus* are also beneficial probiotics.

Probiotics are viable microorganism with several mechanisms of actions such as protection of epithelium barrier, pathogen inhibition via adhesion and colonization on intestinal wall, and production of anti-microbial substances as well as immunomodulation.

There are several evidences reporting the use of probiotics with good properties including *Lactobacillus*, *Bifidobacteria*, *Enterococcus*, *Streptococcus*, *Bacillus*, *Lactococcus* and *Saccharomyces*, which are safe and have been studied for many years[2]. However, the properties of each probiotics are strain-dependent. Therefore, strain selection as well as investigation of other properties of probiotics is necessary. Recently, there are increasing numbers of probiotic studies in food industry and health promotion. Probiotics can be found in local fermented food, raw milk, yogurt, infant feces and flower.

According to Food and Agriculture Organization of the United Nations and World Health Organization[3], probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [3].

Nowadays, probiotics have been used in medicine to compensate and reduce side effects from the use of antibiotics, and as health supplements in many countries around the world. In addition, it is also used in food and animal food products. FAO/WHO announced the required criteria for probiotic standard for probiotic users in 2002[4, 5]. Criteria of probiotic are acid and bile tolerance, survival and colonization in gastrointestinal tract, antimicrobial activity by production of antimicrobial substances, antibody stimulation, antioxidant property, enzyme production and adherence to intestinal wall[6]. According to their properties, probiotics are a good microbiota promoting health.

Microbiota is a microorganism found ubiquitously in human body, especially in the gastrointestinal tract (GI tract). These bacteria are called gut microbiota. This microbes benefits its host in several ways such as helping digestion, detoxification, protection of pathogenic bacteria , helping cross talk between intestinal membrane with immune cell and balancing immune system[7].

Probiotics are good microbiota which play an important roles in human diseases including alcoholic liver disease , obesity, allergy, and inflammatory bowel disease (IBD)[8]. In addition, good microbiota balance microbe in our body, especially in ones who have been used antibiotic for many years and have dysbiosis, an imbalance of microbiota in human body[9, 10]. Dysbiosis of intestinal microbiota occurs when the bad microbiota have number more than a good microbita caused toxin from bad microbiota stimulated an immune cell in the epithelial cell leading to inflammatory bowel disease pathogenesis[11]. For alcohol induced dysbiosis, ethanol changes human microbiome resulting in bacterial overgrowth and reduction of beneficial bacteria. Chronic alcohol consumtion destroys epithelial barrier of intestine causing leaky gut and increases endotoxin in liver cell leading to liver inflammation[12].

Tumor necrosis factor (TNF-α), the main pro-inflammatory cytokines and play a key role in orchestrating the mechanisms of inflammation. The cytokines in this group include interleukin (IL)-1, and IL-6. There are two forms of tumor necrosis factor; TNF-α and TNF-β. TNF-α is produced by macrophages whereas TNF-β is produced by T lymphocytes. After secretion, TNF-α induces other cytokine such as IL-1, IL-6, IL-8 chemokine signaling to recruitment of inflammatory cell and inflammation response.

The level of TNF-α is alleviated in patients with inflammatory conditions including inflammatory bowel disease or IBD, rheumatoid arthritis, alcoholic liver disease, atopic dermatitis, and psoriasis. In addition, the imbalance between good and bad microbiota can be observed in the gastrointestinal tract of these patients. The imbalance between good and bad microbe leads to intestinal wall degradation by toxin of the bad microbe and activation of TNF-α production resulting in inflammation and pathogenesis[13].

It has been shown that *L. plantarum* strain inhibited TNF-α, IL-6, IL-8 secretion in human intestinal epithelial-liked cells (Caco-2) stimulated with lipopolysaccharide (LPS) as model of IBD[14].In animal model, encapsulation of the probiotic *L. kefiranofaciens* reduced colon inflammation[15]. Previous studies reported that *L. johnsonii* and *L. sakei* can attenuated colitis and reduced TNF-α, IL-1, IL-6 expression in colitis mice[16, 17].*In vitro* and *in vivo* studies showed that *L. plantarum* strain activated IL-10 production in macrophages derived from blood monocytes and gut model. Moreover, symptoms of colon inflammation such as weight loss and diarrhea were also attenuated when administered *L. plantarum* strain in dextran sulphate sodium (DSS)-induced colitis rat model[18]. TNF-α inhibition in colon, reduction of oxidative stress and increasing of *Bifidobacterium* spp. in the feces and the gastrointestinal tract were observed in ulcerative colitis mice treated with *L. paracasei*[19]. Recent the study showed that *Bifidobacterium animalis* subsp. *lactis* strain BB12 reduced severity of dextran sulfate sodium-induced ulcerative colitis mice, which reduced disease activity index score, reduced apoptosis in the intestinal epithelial cells by TNF-α production inhibition[20].

World Health Organization (3) reported the continuously increasing rate of heavy alcohol drinking in Thai people from 1961-2010, which majority of alcohol consumption was spirits at about 73%. The mortality rate of male was more than female. Heavy alcohol consumption leading to alcoholism causes liver inflammation and cirrhosis development[21]. Drug is commonly used as a treatment for liver diseases to relief symptoms of patients, and liver transplantation is the last option[22].The alternative treatment for liver disease is using probiotics. Animal model showed that *L. casei* reduced hepatic inflammation and TNF-α, IL-1β production in rat lipopolysaccharide (LPS) and d-galactosamine induced acute liver failure[23]. The previous study shown that *L. rhamnosus* GG mixed diet reduced hepatic inflammation and TNF-α production via inhibition of Toll Like Receptor (TLR) activation in alcohol induced liver injury in mice[24]. Likely the study of *L. rhamnosus* GG (LGG) supplementation alleviated alcoholic liver injury by alcohol induced endotoxin[25].In addition, probiotic lactobacilli have potent of anti-inflammatory properties in human hepatocellular carcinoma cells (HepG-2) stimulated with LPS by

reduced TNF-α, IL-6 secretion[26].Recent study of alcoholic liver injury rats showed that *L. plantarum* C88 reduced liver functional enzymes including alanine transaminase and aspartate aminotransferase (AST) and decreased TNF-α, IL-6 and IFN-γ in serum and inhibition of nuclear factor kappa-B (NF-κB) inflammation signaling pathway[27]. Similarly, treatment with *L. plantarum* and *L. fermentum* reduced ALT and AST level in serum and decreased TNF-α, IL-1β gene expression in acute liver injury mice model induced with carbon tetrachloride[28].

However, there are limited studies of probiotics isolated from Thai people and its inhibitory property on TNF-α production in *in vitro* and *in vivo* model. Therefore, this research aim is to investigate roles of probiotic *Lactobacillus* in the suppression of TNF-α secretion *in vitro* and anti-inflammatory effects in colitis and hepatitis rats. Probiotic *Lactobacillus* might be a potential candidate for ameliorating of intestine and liver inflammation.

Hypothesis

Lactobacillus paracasei strains is capable to inhibit TNF-α secretion *in vitro* as probiotic. *In vivo* study, *Lactobacillus paracasei* reduces intestinal and liver inflammation and modulate beneficial microbiota in gastrointestinal tract.

Objectives

1. To determine the effect of *Lactobacillus paracasei* MSMC39-1 on the suppression of TNF-α secretion in Caco-2 cells and HepG-2 cells.
2. To determine the effect of *Lactobacillus paracasei* MSMC39-1 on the suppression of TNF-α secretion in colitis and hepatitis rat.
3. To study the effect of *Lactobacillus paracasei* MSMC39-1 on aspartate aminotransferase (AST) and alanine aminotransferase levels in colitis and hepatitis rat.

4. To study the effect of *Lactobacillus paracasei* MSMC39-1 on colitis and hepatitis rat by histological investigation.
5. To study the alteration of gut microbiota in colitis and hepatitis rat treated with *Lactobacillus paracasei* MSMC39-1 using Next generation sequencing.

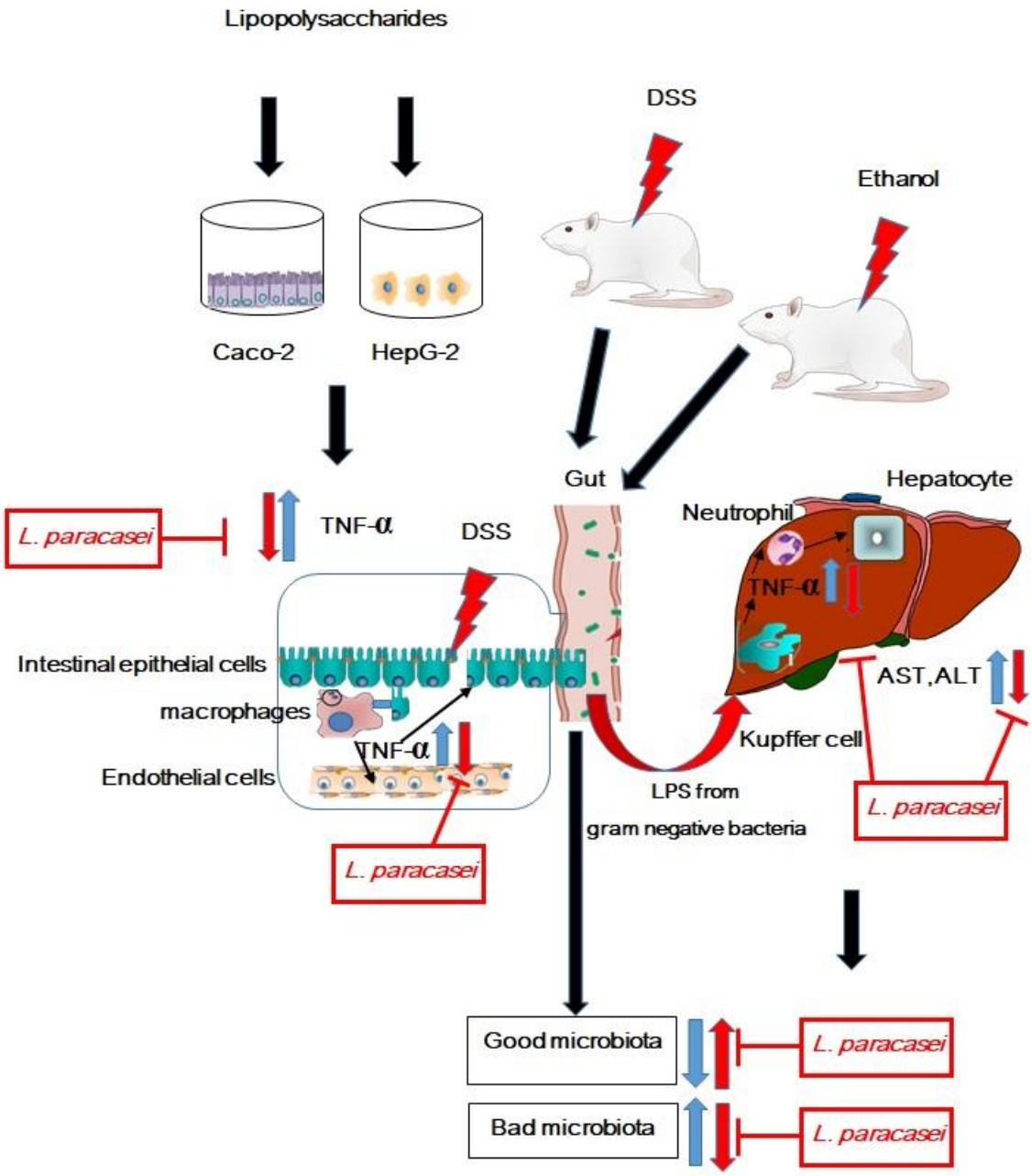

Figure 1 Conceptual framework

CHAPTER 2
LITERATURE REVIEW

1. Genus *Lactobacillus*

Taxonomic of genus *Lactobacillus* is in phylum Firmicutes, Class Bacilli, Order *Lactobacillales* and Family *Lactobacillaceae*. Genus *Lactobacillus* are lactic acid bacteria, gram positive bacteria, rod shape, non-sporulating, facultative bacteria, produced lactic acid, and the most common probiotic because of its safety according to the criteria of Generally Recognize as Safe (GRAS) by Food and Drug Administration (FDA or USFDA). The major groups of genus *Lactobacillus* by 16S RNA gene *L. sakei* and *L. salivarius*. Lactobacilli have probiotic properties such as acid-bile tolerant in gastrointestinal tract (GI tract), adhesion to intestinal epithelial cell, anti-microbial activity and immunomodulation. They can be found mainly in GI tract, human feces, breast milk, fermented milk, cheese, yogurt, traditional foods, preservative foods, ice-cream and fruit juice. *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. casei*, *L. rhamnosus* and *L. paracasei* have immunomodulation activity[29, 30]. Recent study in *n vitro* demonstrated that *L. paracasei* have high adhesion activity, tolerate to acid, bile and have viability in conditions of GI tract.[31] *L. paraplantarum* have potent high adhesion activity and immunomodulation by reduced pro-inflammatory gene expression.[32] In 2018, Liu Y and coworker, showed that treatment with probiotic VSL #3, *L. reuteri*, *L. rhamnosus* GG, *L. johnsonii, S. boulardii* reduced inflammatory bowel disease in ulcerative colitis and Crohn's disease patient[33].

2. Probiotics

Probiotic was first discovered by Elie Metchnikoff (Figure 2)[34], who received Nobel Prize in 1908. He showed that yogurt fermented with Bulgarian bacillus are benefit to human health by prolonging life expectancy.

Definition of probiotics is defined as "live microorganisms that when administered in adequate amounts, confer a health benefit on the host" by Food and Agriculture Organization of the United Nations and World Health Organization [3]. Guidelines of

probiotics for using in the food such as identification of species and strains, screening in *in vitro* including resistance to acid, resistance to bile, adhesion to epithelial cell, colonization ability, anti-pathogenic activity by producing anti-microbial substance, production of bile salt hydrolase, modulation of immunity, multiplication in large scale and safety according to Generally Recognized as Safe (GRAS). These criteria of probiotics were summarized in Table 1. [3] Probiotics can tolerate acid and bile then survival and colonize in gastrointestinal tract for promoting health. Present, criteria selection of probiotics in functional properties are anti-cancer, anti-cholesterol, anti-depression and anti-anxiety, anti-obesity, stimulated of immunity ability and molecule secretion[35]. Sources of probiotics are found in fermented foods, beverage,[36] human feces,[37] fermented milk,[38]breast milk,[39] food supplement and dairy products[40].

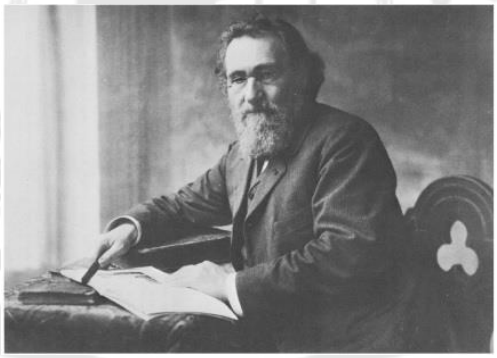

Figure 2 Elie Metchnikoff (1845-1916).

Source: Mackowiak PA (2013) . Recycling metchnikoff: probiotics, the intestinal microbiome and the quest for long life p. 2.

Table 1 Criteria for probiotic strains

Desirable characteristics of an ideal probiotic microorganisms
Human origin
Generally recognized as safe (GRAS)
Acid and bile tolerance
Adherence to gut epithelial cell
Colonization in the gastrointestinal tract
Antimicrobial substances production
Immune responses modulation
Amenable to large scale fermentation and commercial production

3. Strains used as probiotics

Probiotics according FAO/WHO is mainly lactic acid bacteria including genus mainly genus *Lactobacillus* and *Bifidobacterium*. *Lactococcus*, *Saccharomyces*, *Enterococcus*, *Pediococcus*, *Streptococcus*, *Leuconostoc*, *Bacillus* and *Escherichia* strains have been used as health beneficial also. Summary of probiotics with health benefit from database updated in 2014 is presented in Table 2.[41] *Lactobacillus casei* Shirota have been shown immunomodulatory and reduced rheumatoid arthritis.[41] *Bifidobacterium bifidum*, *B. longum* and *B. breve* were attenuated necrotizing enterocolitis in children.[41] *Saccharomyces boulardii* was treated pseudomembrane colitis.[41]

Table 2 Strains used as probiotics updated in 2014

Genus	Species	Recently published probiotic on health (strain specific)
Lactobacillus	*L. rhamnosus*	- *L. rhamnosus* CRL1505 reduced pulmonary damage. - *L. rhamnosus* GG was reduced atopic dermatitis. - *L. rhamnosus* HN001 and *L. rhamnosus* GG were reduced the risk of allergic disease. - *L. rhamnosus* GG was anti-diabetic potential (various strains from human infant faecal samples) and prevented necrotizing enterocolitis in newborns. - *L. rhamnosus* GR-1 was prevented bacterial vaginosis. - *L. rhamnosus* CGMCC1.3724 alleviated obesity women. - *L. rhamnosus* GG treated acute gastroenteritis in children. - *L. rhamnosus* GG and *L. rhamnosus* ATCC 53103 were reduced rhinovirus infections in preterm infants. - LGG was protect human colonic muscle damage from induced by lipopolysaccharide.
	L. acidophilus	- Reduced infections in children in irritable bowel disease.
	L. plantarum	- *L. plantarum* NRRL B-4496 was prevented endotoxin production, antifungal activity and reduced irritable bowel disease.
	L. casei	- *L. casei* Lcr35 and *L. casei* Shirota were treated constipation in adults and *C. difficile*-associated diarrhea. - *L. casei* Lcr35 was restored vaginal flora in bacterial vaginosis patient. - *L. casei* Shirota was reduced symptoms of irritable bowel syndrome and diarrhea of antibiotic-associated diarrhea in geriatric patients.

Table 2 (continued)

Genus	Species	Recently published probiotic on health (strain specific)
		- *L. casei* Shirota have immunomodulatory activity. - *L. casei* 01 was improved rheumatoid arthritis. - *L. casei* CRL-431 was protected against *Salmonella* infection. - *L. casei* IMV B-7280 was prevented of *Salmonella*-induced synovitis and treated intravaginal staphylococcosis.
	L. delbrueckii subsp. *bulgaricus*	- *L. delbrueckii* subsp. *bulgaricus* 8481 was supplemented of yogurt in antibiotic resistance and enhanced systemic immunity in elderly. - Against *E. coli* growth. - Modulate in brain activity.
	L. brevis	- *L. brevis* KB290 was protected in bile salt tolerance. - *L. brevis* CD2 was reduced plague acidogenicity.
	L. johnsonii	- *L. johnsonii* MH-68 was reduced gastritis, *H. pylori* infection and protected against respiratory insults. - *L. johnsonii* F0421 was inhibited of *S. sonnei* activity. - *L. johnsonii* EM1 was treated perennial allergic rhinitis in children.
	L. fermentum	- *L. fermentum* RC-14 was prevented bacterial vaginosis. - *L. fermentum* ATCC 11739 was blocked pathogenic microorganisms to adherence on vaginal epithelium and anti-staphylococcal action. - *L. fermentum* NCIMB 5221 was reduced insulin resistance and hypercholesterolemia.

Table 2 (continued)

Genus	Species	Recently published probiotic on health (strain specific)
	L. reuteri	- *L. reuteri* NCIMB 30242 was reduced low-density LDL cholesterol. - *L. reuteri* ATCC 55730 was treated acute gastroenteritis in children. - *L. reuteri* ATCC 55730 and *L. reuteri* DSM 17938 were reduced diarrhea in children and infant colic. - *L. reuteri* DSM 17938 was reduced gastrointestinal disorders in infants. - *L. reuteri* DSM 17938 reduced sepsis and duration of hospital stay in infants.
Bifidobacterium	*B. infantis*	- Reduction of irritable bowel syndrome and necrotizing enterocolitis.
Bifidobaterium	*B. animalis* subsp. *lactis*	- *B. animalis* subsp. *lactis* DN-173 010 was improved constipation. - *B. animalis* subsp. *lactis* DN-173 010 was reduced febrile urinary tract infections in children, modulate brain activity, necrotizing enterocolitis, microbial in dental plaque in infant. - *B. animalis* subsp. *lactis* MB 202/DSMZ 23733 was reduced cholesterol. - *B. animalis* subsp. *lactis* BI-04 was reduced the risk of upper respiratory illness.
	B. bifidum	- *B. bifidum* MB 109/DSMZ 23731 was reduced acute diarrhoea in children, necrotizing enterocolitis in infants and cholesterol.

Table 2 (continued)

Genus	Species	Recently published probiotic on health (strain specific)
	B. longum	- *B. breve*, *B. infantis*, *B. bifidum*, *B. longum* were prevented necrotizing enterocolitis, reduced diarrhea, necrotizing enterocolitis with Bifidobacteria cocktail.
	B. breve	- *B. breve*, *B. infantis*, *B. bifidum* and *B. longum* were prevented necrotizing enterocolitis in newborns and reduced necrotizing enterocolitis with Bifidobacteria cocktail. - *B. breve* MB 113/DSMZ 23732 reduced total cholesterol.
Saccharomyces	*S. boulardi*	- Treatment of travellers' diarrhoea, diarrhoea duration regardless, irritable bowel syndrome, moderate ulcerative colitis, pseudomembrane colitis infection, acute gastroenteritis in children.
Lactococcus	*L. lactis* subsp. *lactis*	- *L. lactis* subsp. *lactis* KLDS4.0325 was treated in antibiotic-associated diarrhea, adhesion to vaginal epithelial cells. - *L. lactis* subsp. *lactis* CV56 nisin production. - *L. lactis* subsp. *lactis* ATCC 11454 was modulated brain activity, against *C. difficile* and have potent probiotic properties.
Enterococcus	*E. durans*	*E. durans* LAB18s have antioxidant activity, adherence to colonic tissue and anti-inflammatory activity.

Table 2 (continued)

Genus	Species	Recently published probiotic on health (strain specific)
	E. faecium	-Treatment of antibiotic-associated diarrhea and effective in animal.
Pediococcus	*P. acidilactici*	-*P. acidilactici* UL5 produced pediocin with antimicrobial activity and probiotic properties. -*P. acidilactici* BA28 was eliminated bacteriocin of *H. pylori* infections.
Leuconostoc,	*L. mesenteroides*	- Produced leucoin, pepsin, bile salts and have probiotic properties.
Bacillus	*B. coagulans*	- *B. coagulans* ATCC PTA-11748 was treated antibiotic-associated diarrhoea and bacterial vaginosis. - *B. coagulans* GandenBC30 supported immunology in children.
	B. subtilis	- *B. subtilis* R0179 have effective in animal probiotic; treated in diarrhea and *H. pylori* inhibition and nitric oxide production.
Escherichia	*E. coli* Nissle 1917	- Constipation in adults, IBD and gastrointestinal disease treatment.

3.1 *Lactobacillus paracasei*

Lactobacillus paracasei is in *L.casei* group similar to *L. rhamnosus*, gram positive bacteria, rod shape, size 0.7-1.1 x 2.0-4.0 µm (Figure 3), non motile and it was an application in food products such as cheese, fermented milk, fermented food and especially, used as probiotic. The major in the human health effects of *L. casei* group including immunomodulation, allergy prevention, colitis prevention, intestinal barrier function protection, liver injury prevention, *Helicobacter pylori* inhibition and gut microbiome modulation (Table 3)[42-47]

Table 3 The effects of *L. casei* group in the human health

Strain	Effect
Lactobacillus casei Shirota	Modulation of immune system Diabetes mellitus protection Against multi-drug resistant *Salmonella* protection
Lactobacillus rhamnosus Lcr35	Asthma prevention Anti-inflammatory effect in intestinal epithelial cells
Lactobacillus casei DN-114 001	Duration of respiratory infections reduction Barrier function protection Incidence of illness reduction
Lactobacillus paracasei ST11	Allergic rhinitis treatment Colitis prevention
Lactobacillus rhamnosus HN001	Allergy attenuation
Lactobacillus paracasei F19	Decreasing of fat storage Liver injury attenuation
Lactobacillus casei ATCC 334	Anti-arthritic
Lactobacillus rhamnosus GG	Anti-biofilm of *Streptococcus mutans* in saliva Immune function modulation Against ochratoxin protection
Lactobacillus casei ATCC 393	*Helicobacter pylori* inhibition Gut microbiome modulation

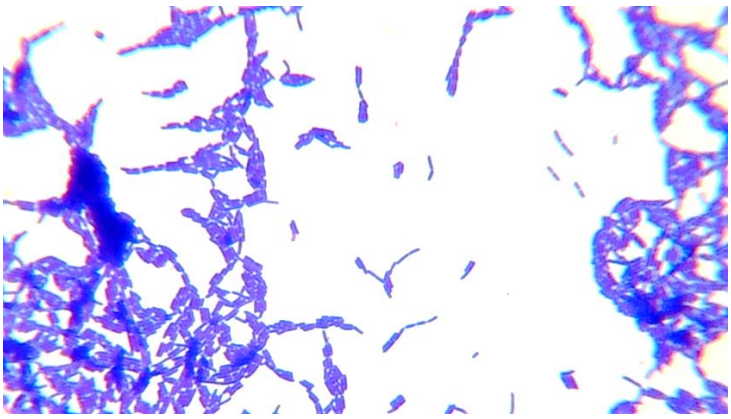

Figure 3 Morphology of *L. paracasei*.

3.2 *Weissella confusa*

Weissella confusa or *Lactobacillus confusus* is Family Leuconostocaceae, lactic acid bacteria, gram positive coccobacilli (Figure 4). Application of *Weissella confusa* in fermented food such as sourdough, daily products, pasta, phamarceutical, cosmetics, anti-oxidant and anti-microbial activity[48-51]. Exopolysaccaride from *Lactobacillus confusus* was immunomodulation such as iduced TNF-α, IL-1β and IL-6 secretion in RAW264.7 macrohage cells[52].

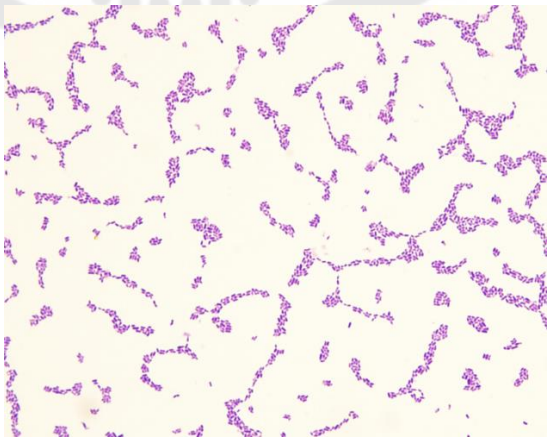

Figure 4 Gram stain of *Weissella confusa*

4. Mode of action of probiotics

Mechanisms of probiotics are intestinal epithelium protection by increase secretion of mucus or production of anti-microbial substances such as reuterin, bacteriocin, defensin.[53] Similarly, previous study showed that probiotic *L. salivarius* producing bacteriocin.[54] Supplementation of *L. reuteri* produced reuterin inhibited *Listeria monocytogenes* and *Escherichia coli* in cheeses.[55] Probiotics can modulate immune response such as stimulate dendritic cell present antigen to T cell produce interferon and cytokine and stimulate B cell produce antibody. Treatment with *Bifidobacterium infantis* improved colitis by activated dentritic cell in mice.[56] *L. gasseri* potentialy induced B cell produced IgA antibody in small intestine of mice.[57] Probiotic *L. acidophilus* supplementation have potent inhibited TNF-α, IL-1β, IL-6 cytokine and IL-8 chemokine production in mice colitis model[58]. Last but not least, probiotics can inhibited pathogen to adherence on epithelium cells via production of anti-microbial factors to inhibit pathogen adhesion, or probiotics have high adhesion activity (Figure 5). In addition, there are several evidences showed that mixed strains of probiotics inhibited biofilm formation of *E. coli* and inhibited *E. faecalis*[59]. *L. pentosus* and *L. plantarum* exhibited high adhesion activity in colon cancer cell line (Caco-2 cells)[60]. Recent study showed that *L. plantarum* inhibited adhesion of *Salmonella enterica* in colorectal carcinoma cell line (HCT-116 cell line)[61].

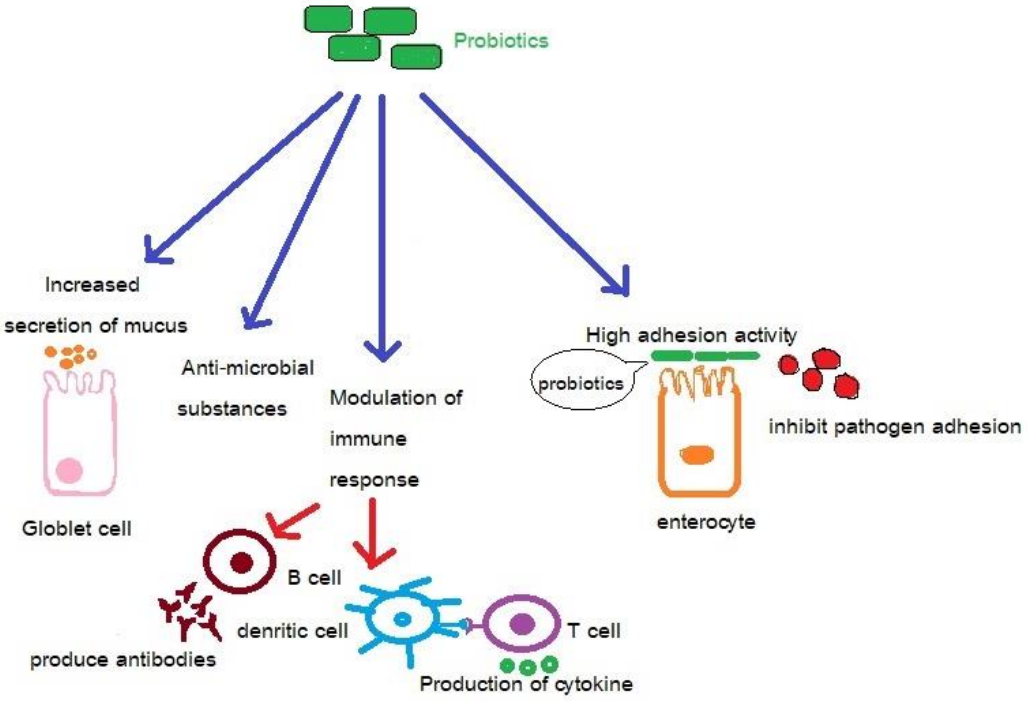

Figure 5 Diagram of the cross-talk between probiotics and intestinal mucosa.

Major action of probiotics include the protection of intestinal barrier function by activated production of mucus, antimicrobial peptides, immunomodulation and anti-microbial activity by competitive with pathogen for adherence to intestinal cell.

Moreover, probiotics isolated from human origins are beneficial for our health and well-being. Probiotics exhibit anti-obesity, anti-inflammatory, anti-cancer and anti-allergy properties. In addition, they also can tolerate storage and transportation processes. Probiotics with anti-microbial properties are *E. durans*,[62] *L. curvatus* and *L. sakei*,[63] which inhibited *Listeria monocytogenes* growth. *Lactobacillus* strains isolated from yogurt show anti-microbial activity including *E. faecalis*, *S. aureus*, *Pseudomonas aerogenosa*, *K. pneumonia*, *S. typhi* and *E. coli*[64]. Furthermore, *L. paraplantarum* isolated from Korean food induced TNF-α gene expression in Raw macrophage 264.7 cells and *L. rhamnosus* showed anti-oxidant activity in *in vitro*.[65, 66] Meanwhile, *L. plantarum* and *B. longum* decreased TNF-α secretion in THP-1 cell and human skin samples, respectively.[67, 68]

5. Probiotics and microbiota

Human microbiome project (HMP) is a project studying on all microorganisms in human body which are related to human diseases. Microorganism lives in human body as normal flora, non-pathogenic which is called “microbiota”. Microbiota is defined as microorganism including bacteria, virus and yeast. At present, modern techniques including shotgun, 16S RNA gene sequencing are used as a characterization tool of the microbiota. In human body, most of microbiota live in the gut. There are more than trillion species of gut microbiota, and its number depends on environmental and genomic factors of host. As natural birth infant we receive microbiota from our mother’s vagina and infant with cesarean section, typically microbiota found on skin.[69]

At present, several evidences supported the roles of microbiota in health promotion. From infant to adult life, microbiota can be found in vagina, skin, and stool. It also can be transferred into our body by touching, food consumption and environment. Therefore, there are numerous microbiota species in our body, especially intestinal microbiota that are composed of good and bad microorganism for balancing body[70]. Probiotics are a good microbiota can promote health such as producing anti-microbial substances or compete pathogen adherence to intestinal epithelial cell[71], reduced cholesterol level[72] and anti-cancer properties[73].

The role of intestinal microbiota is to the maintain balance of GI tract. When intestinal microbiota is imbalance resulting in "dysbiosis" causing disruption of intestinal barrier which, toxin through into the epithelial cell and stimulate immune cell for the production of pro-inflammatory cytokine and resulted in intestinal inflammation. There are several factors caused dysbiosis including genetic, antibiotic, diet and gender. In addition, chronic dysbiosis enhances the risk of colitis and hepatitis pathogenesis.[74] Treatment of colitis used an antibiotic, probiotic, prebiotic and fecal transplantation as shown in Figure 6. Hepatitis treatment used a vitamin, drug, liver transplantation and probiotic.[75]

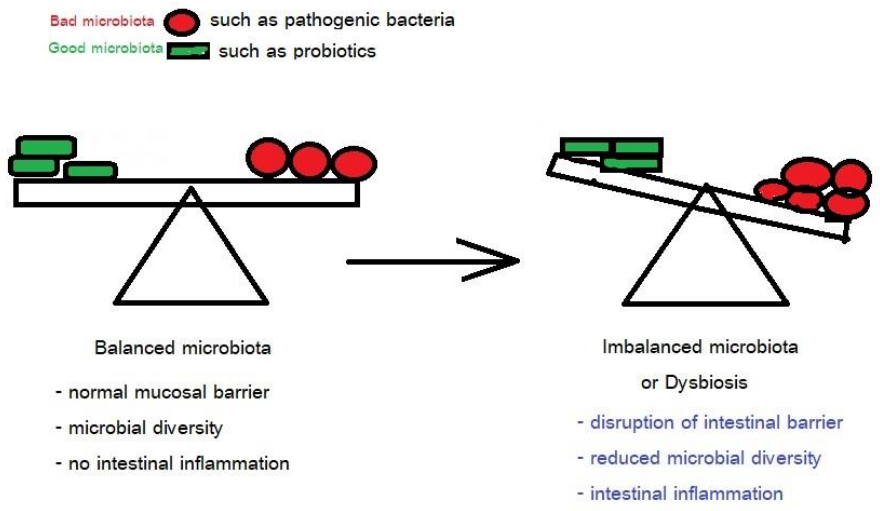

Figure 6 Potential therapeutic strategies for modulating intestinal dysbiosis.

6. Tumor necrosis factor

Tumor necrosis factor (TNF-α) is the main pro-inflammatory cytokine, which is produced when infectious pathogen or toxin. Tumor necrosis factor alpha (TNF-α) plays a key role in inflammation process. TNF-α is the first cytokine secrete when body response to the inflammation process and induced other cytokine such as interleukin (IL)-1 and IL-6 and induce phagocyte cells for phagocytosis pathogen or toxin until tissue inflammation. TNF is produced by activated macrophages, lymphocytes, dendritic cells and other cells. Tumor necrosis factor are divided into two forms which are TNF alpha (TNF-α) and TNF beta (TNF-β). Mechanism of TNF-α in gene expression, TNF-α binds to TNF receptor 1 and activates cascades via Ikappa B (I**K**B) kinase (IKK)/NF-**K**B and mitogen-activated protein kinase (MAPK)/AP-1 pathways, leading to gene expression of pro-inflammatory cytokines, chemokines, cell survival and growth factor [(76)].

Roles of TNF-α in innate immunity are activation of endothelial and neutrophil cells for secretion of pro-inflammatory cytokine and chemokine at local inflammation. For

systemic responses, TNF-α action on hypothalamus and increase body temperature caused fever. TNF-α, IL-1 and IL-6 can enhance protein synthesis in liver, and support leucocyte production in bone marrow. TNF-α causes cardiovascular disease, endothelial cell alteration caused thrombosis, myocardial contraction was inhibited and insulin resistance in metabolic disease[77] (Figure 7-8). Serum TNF-α level is elevated in several inflammatory diseases including IBD, rheumatoid arthritis and psoriasis. Therefore, anti-TNF drug is another option to treat these diseases.[78, 79]

Anti-TNF-α treatment was used in clinical trials to treat inflammatory disorder such as IBD, rheumatoid arthritis, psoriasis.[62] In addition, increasing of TNF-α level may cause ethanol induced dysbiosis in alcoholic hepatitis leading to liver inflammation.[61]

There are several studies reported using probiotic to modulate TNF-α. Heat-killed lysate cells of *L. plantarum* exhibited immunomodulation property in mouse splenocytes[80].Similarly, Gomez-llorente C and coworker showed cell free supernatant of *L. rhamnosus* inhibited pro-inflammatory cytokine in dentritic cells[81]. Live and heat-killed *L. rhamnosus* have potent modulation of TNF-α, IL-6 and IL-10 secretion.[82] In osteoarthritis (OA) model, *L. acidohilus* reduced TNF-α and IL-6, and increased IL-10 expression and reduced pain of OA rat.[83]

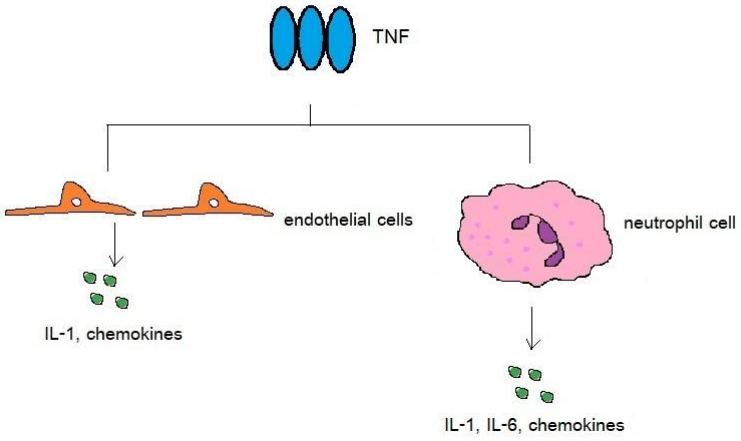

Figure 7 Local action of cytokines in inflammation.

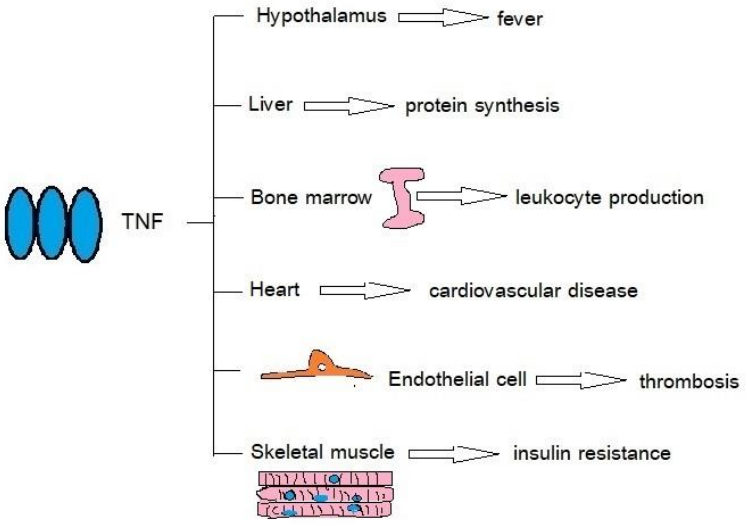

Figure 8 Systemic action of cytokine in inflammation.

7. Role of probiotics in inflammatory diseases

7.1 Inflammatory bowel disease (IBD)

Inflammatory bowel disease (IBD) is a chronic intestinal inflammation, has two forms which are Crohn's disease (CD), an inflammation of small intestine, and ulcerative

colitis (UC), a colon inflammation. There are several factors causing IBD including genetic, extraneous environment, dysbiosis in intestinal microbiota and immune response.[84] Patients with IBD have increase in pro-inflammatory cytokine level such as TNF-α, IL-1, and IL-17.[85]

Pathogenesis of IBD is caused by dysbiosis of intestinal microbiota leading to a reduce in bacteria and fungi diversity, and an increase in virus diversity. Inflammatory bowel disease is causes by intestinal barrier dysfunction allowing endotoxin released from bad microbiota to activate intestinal macrophages to secrete pro-inflammatory cytokine resulting ulcer of mucosal and pathogenesis Figure 9. Treatments of IBD are anti-TNF-α treatment, fecal transplantation and probiotic treatment.[86, 87] Administration of *L. plantarum* isolated from feces alone or with prebiotic potentially reduced intestinal inflammation by inhibition of TNF-α secretion in colitis rat.[88] Previous study showed that *L. casei* combined with superoxide dismutase reduced inflammation in colitis mice model.[89] Moreover, live and heat-killed antigen of *L. casei* can regulate regulatory T cell responses in colitis model.[90] The reduction of TNF-α production was observed in necrotizing enterocolitis rat treated with combine conditioned media of *L. acidophilus B. infantis* and *L. plantarum*.[91] Moreover, *L. plantarum* also reduced TNF-α secretion in colitis model.[92] For *in vitro* and *in vivo* colitis models, encapsulate *Lactobacillus rhamnosus* GG and extract of *L. brevis* attenuated colitis and inhibited pro-inflammatory cytokine production.[93, 94] Moreover, probiotic *L. curvatus* isolated from fermented food reduced IBD pathogenesis.[95] In *in vitro*, *Saccharomyces cerevisiae* and *L. plantarum* in fermented rice exhibited potent antioxidant and anti-inflammatory effects and effectively reduced score in IBD.[96] Study of probiotic *L. plantarum* fermented in ginseng reduced colitis and decreased pro-inflammatory cytokine in plasma and intestinal tissue of mouse model.[97] Soy bean fermented with *E. faecium L. helveticus* and *B. longum* reduced colitis and increase genus *Lactobacillus* and *Bifidobacterium* in GI of dextran sodium sulphate (DSS) induced colitis rat.[98] *L. fermentum* reduced symptoms of colitis by decreasing inflammatory cells and inhibited pro-inflammatory cytokine production such as IFN-γ, IL-12, TNF-α and IL-6.[99] Recent studies in IBD mice induced with DSS suggested that

Lactobacillus lactis[100] *L. plantarum*,[101] *L. acidophilus*,[102] *L. gasseri*,[103] *E.coli* [104] and *B. breve*[105] exhibited potential anti-inflammation effect and protected colon damage. Polyphosphate of *L. brevis* decreased TNF-α, IL-1β, fibrosis and inflammation of intestine in trinitrobenzene sulfonic acid (TNBS) and DSS induced colitis mouse model.[106] In addition, *L. plantarum*,[107] *L. kefiranofaciens*,[15] mixture of *B. longum* and *L. brevis*,[108] *L. johnsonii*,[16] *B. bifidum*[109] modulated pro-inflammatory cytokine production and decreased intestinal inflammation in this model. However, *L. rhamnosus* was the only strain which increased genus *Bifidobacterium* microbiota in the gut of TNBS and DSS induced colitis rat.[110]

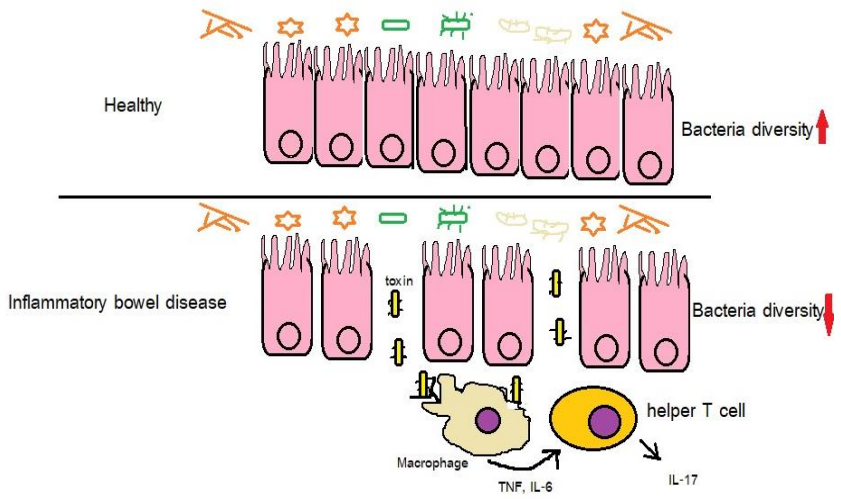

Figure 9 Gut microbiota alteration and immune responses in IBD.

7.2 Alcohol hepatitis

Alcohol hepatitis caused by chronic alcohol drinking exhibits fat and toxin accumulation, intestinal barrier disruption and pro-inflammatory cytokine stimulation resulting in liver inflammation causing dysbiosis. Ethanol consumption reduces the number of good microbiota leading to releasing of lipopolysaccharide (LPS) or toxin from bad microbiota[61]. This toxin then binds to toll like receptor 4 (TLR-4) activated kupffer cells to secret pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6, and generates

reactive oxygen species by induced NF-κB/JNK/Ap-1 pathway (Figure 10). In the addition, *Lactobacillus spp*. reduced TNF-α secretion, inhibited TNF-α, IL-1β, and IL-6 gene expression in the hepatic tissue of rat induced liver fibrosis.[111] In the alcoholic liver disease model, combination of probiotic *L. rhamnosus* and *L. acidophilus* reduced toll like receptor-4 and IL-1β level in the liver tissue.[112] *L. rhamnosus* and *L. acidophilus* decreased levels of TNF-α, IL-1β and TLR-4 expression as well as increase anti-inflammatory cytokine (IL-10) in alcoholic hepatitis mouse model.[113]

Treatments of alcoholic hepatitis are administration of vitamin supplement, corticosteroids (anti-pro-inflammatory cytokine), and the last option is liver transplantation. Alternative treatment of alcoholic hepatitis is using probiotic to modulate microenvironment in gastrointestinal tract and immune responses.[61] Treatment of *Lactobacillus fermentum* restored liver damage in ethanol induced alcohol liver disease.[114] *In vitro* and *In vivo* studies showed that heat-killed of *L. salivarius* and *L. johnsonii* reduced aspartate transaminase (AST), alanine transaminase, and gamma-glutamyl transferase (γ-GT) levels and oxidative stress in hepatocellular carcinoma cell line.[115] Reduction of γ-GT enzyme was also observed in alcohol induced liver injury mouse model.[116] Furthermore, supplement of probiotic improved liver fibrosis by reduced inflammation and oxidative stress via mitogen-activated protein kinase (MAPK) signaling pathway in model of liver fibrosis rat.[116]

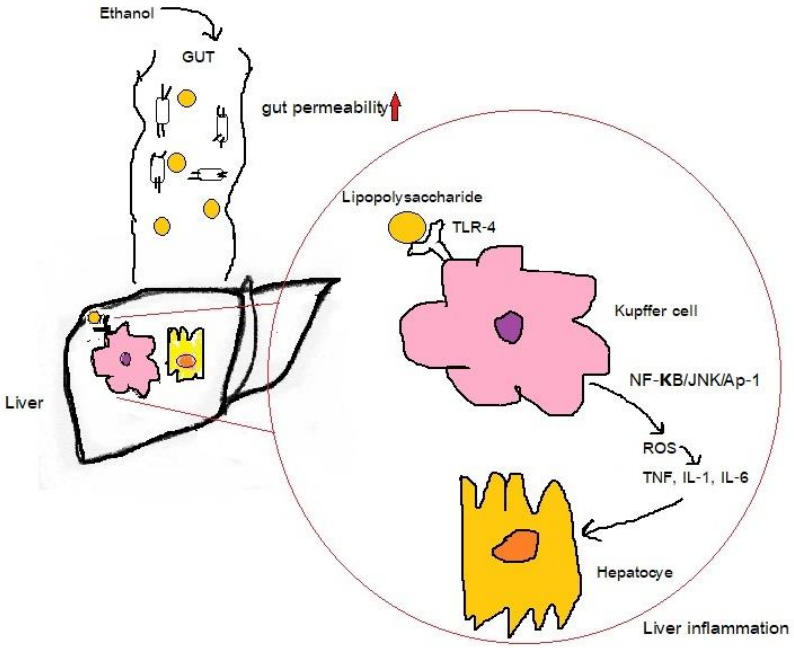

Figure 10 Ethanol induced gut permeability and inflammatory process cascade.

7.3 Atopic dermatitis (AD)

Atopic dermatitis, which is mainly found in children, is a chronic skin inflammation caused from food allergy, asthma, and dysbiosis. Anti-histamine grugs and probiotics are common treatments for atopic dermatitis.[117] *Lactobacillus acidophilus, B. bifidum, L. casei, L. salivarius* were attenuated dermatitis in pediatric.[118] Previous study in clinical trial demonstrated that *B. animalis* subsp *lactis* LKM512 improved atopic dermatitis.[119] Probiotic *Lactococcus chungangensis* reduced atopic dermatitis.[120] Recent evidence showed that *L. rhamnosus* was safe to treat atopic dermatitis in children age between 4-48 months old.[121]

7.4 Psoriasis

Psoriasis, a skin inflammation with hyperplasia of keratinocyte, increase IL-17 level in serum and skin lesion. *Lactobacillus pentosus* reduced thick tissue of epidermal, serum pro-inflammatory cytokine levels such as IL-17, TNF-α, IL-22, and IL-23 in psoriasis mouse model. This strain was also reduced IL-17/IL-22 in the spleen cells.[122]

7.5 Rheumatoid arthritis

Rheumatoid arthritis is caused by intestinal microbiota dysbiosis leading to leaky gut and toxin transfer to blood circulation. Rheumatoid arthritis patients have increases immune complexes at join areas.[123] Treatment with *L. casei* and *L. acidophilus* reduced paw inflammation, increased protein and calcium levels in serum of mouse arthritis model.[124] In clinical trial, supplementation of probiotic *L. casei* increased IL-10 and reduced TNF-α, IL-6 in the serum and attenuated arthritis in arthritis patients.[125] *In vivo* study showed that *L. helveticus* inhibited pro-inflammatory cytokine productions such as TNF-α, IL-17, and IFN-γ as well as stimulated anti-inflammatory cytokine productions such as IL-10.[126]

CHAPTER 3
RESEARCH METHODOLOGY

Chemical and materials

1. deMan-Rogosa-Sharpe (MRS) media (Oxoid, Basingstoke, Hampshire, UK)
2. Glycerol (Merck, USA)
3. Cell lines: HepG-2 cell (ATCC, HB 8065)
4. Cell lines: Caco2 cell (ATCC, HTB 37)
5. DMEM (Gibco-Invitrogen, Carlsbad, CA, USA)
6. Lipopolysaccharide from *E. coli* (Sigma, USA)
7. Albumin bovine fraction V powder (Sigma, USA)
8. Anerobic gas package (MGC, Japan)
9. Fetal bovine serum (Gibco-Invitrogen, USA)
10. Penicillin-Streptomycin (Gibco-Invitrogen, USA)
11. Flat-bottomed tissue culture plates (Corning, USA)
12. Hemocytometer (Hausser Scientific, USA)
13. 25% Trypsin (Gibco-Invitrogen, USA)
14. Counter (Fisher Scientific, USA)
15. ELISA plate: 96- well plate: High binding (Nunc, USA)
16. Recombinant human TNF-α (R&D Systems, USA)
17. Recombinant Rat TNF-α (R&D Systems, USA)
18. Human TNF-α ELISA kit (R&D Systems, USA)
19. Rat TNF-α ELISA kit (R&D Systems, USA)
20. BioTek® Synergy™ HT (Multi-Detection Microplate Reader, USA)
21. Tween 20 (merck, USA)
22. EMB (Himedia, India)
23. BSM (Himedia, India)
24. 0.22 µm pore size filter Millipore (MA, USA)
25. Syringes (NIPRO, Thailand)

26. Isoflurane (Piramal clinical care, USA)
27. ultrasonic homogenizers (Sonopuls, Bandelin, Germany)
28. Paraformaldehyde (Merck, USA)
29. 6-well plate, 24-well plate (corning, USA)
30. Autoclave (J.P. selecta, Spain)
31. Biosafety cabinet (Nuaire, USA)
32. Spectrophotometer (Human, Korea)
33. Whitley Jar Gassing System (UK)
34. pH meter (Thermo Scientific, USA)
35. Anaerobic jar (Mitsubishi, Japan)
36. Calcium carbonate (Merck, USA)
37. Vacuum concentrator (Christ, Germany)
38. Tetramethylbenzidine (R&D Systems, USA)
39. H2O2 (Merck, USA)
40. Water bath (J.P. selecta, Spain)
41. Dextran sulfate sodium

Methods

1. *Lactobacillus* strains

Probiotic *Lactobacillus* strains were used in this study included *Lactobacillus paracasei* MSMC 39-1, *L.casei* MSMC39-3 and *Weissella confusa* MSMC57-1. These strains were isolated from newborn feces which inhibited TNF-α secretion in THP-1 monocytic cell line[127]

2. Cultivation of probiotic *Lactobacillus* strains

Lactobacillus paracasei MSMC 39-1, *L.casei* MSMC39-3 and *Weissella confusa* MSMC57-1 in -80 °C cultured stock were inoculated in deMan-Rogosa-Sharpe (MRS) broth (Oxoid, Basingstoke, Hampshire, UK). The samples were incubated at 37 °C for 48 h, anaerobic condition generated by using a generate gas (Whitley Jar Gassing System, UK) in an anaerobic jar (Mitsubishi, Japan). The culture *Lactobacillus* spp. were streaked on MRS agar supplemented 0.3 % calcium carbonate (Merck, USA) and incubated at 37 °C for 48 h under anaerobic condition. The overnight culture of *Lactobacillus* spp. were picked up as a single colony and added in MRS broth, incubated 37 °C , 48 h, anaerobic condition for next experiments.

3. Preparation of probiotic *Lactobacillus* strains

3.1 Probiotic culture supernatant

Lactobacillus paracasei MSMC 39-1, *L.casei* MSMC39-3 and *Weissella confusa* MSMC57-1 were cultured on MRS agar and incubated at 37°C under anaerobic condition for 48 h. *Lactobacillus* spp. were picked up as a single colony in MRS broth and incubated at 37°C, anaerobic condition for 48 h. *Lactobacillus* spp. were adjusted to final concentration at 10^9 CFU/ml[127] by optical density at 600 nm. The supernatant of *Lactobacillus* spp. were separated by centrifugation and filtered with 0.22 μm and were concentrated by vacuum concentrator (Christ, Germany) at 40 °C for 4-6 h. Samples pellet were resuspended in serum-free-medium DMEM and kept at -20 °C until used.

3.2 Viable cells

The single colony of *Lactobacillus* strains were cultured in MRS broth and incubated at 37°C under anaerobic condition. The samples were adjusted to the final concentration at 109 CFU/ml[126] by measured optical density at 600 nm and washed twice with 1x phosphate buffer saline (PBS). *Lactobacillus* strains were re-suspended with PBS and used immediately for TNF-α inhibition test in cell line. The candidated strain was prepared in viable cells pattern and treated in hepatitis and colitis animal experiments.

3.3 Heat killed cells

Lactobacillus strains were cultured on MRS agar, anaerobically incubated at 37°C for 48 h. The single colony of *Lactobacillus* strains were cultured in MRS broth, incubated at 37°C in anaerobic condition for 48 h. *Lactobacillus* strains were adjusted to the final concentration at 10^9 CFU/ml,[127] centrifuged and washed twice with PBS. The pellets were re-suspended in PBS and boiled at 85 °C for 1 h, and kept at -20°C until used. The viability of samples were re-checked by streaking on MRS agar, and anaerobically incubated at 37°C for 48 h.

3.4 Sonicated cells

Lactobacillus strains were cultured on MRS agar incubated 37 °C anaerobic for 48 h. *Lactobacillus* spp. were picked up and a single colony was cultured in MRS broth and incubated at 37 °C in anaerobic condition for 48 h. *Lactobacillus* spp. were adjusted to the final concentration at 10^9 CFU/ml[127] and were centrifuged and pellets were re-suspend in serum-free medium DMEM. Sonication of *Lactobacillus* spp. were set speed at 85-88% for 7.10 min by ultrasonic homogenizers (Sonopuls, Bandelin, Germany) and kept at -20 °C until use.

4. Preparation of cell culture

Two cell lines including colorectal adenocarcinoma cell (Caco-2 cells) and hepatocellular carcinoma cell (HepG-2 cells) were used in this experiment. Caco-2 cells (ATCC, HTB 37) and HepG2 cell (ATCC, HB 8065) was purchased from American Type Culture Collection (ATCC). The $Caco_2$ cell was cultured in high glucose DMEM (Gibco-Invitrogen, Carlsbad, CA, USA) and HepG2 cell was cultured in low glucose DMEM which,

both cell lines was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin (100 units/ml) streptomycin (100 mg/ml), incubated in 37°C incubator, 98% humidified and 5% CO_2.

4.1 The study of probiotic *Lactobacillus* strains in the suppression of TNF-α secretion in Caco-2 cells

The Caco-2 cell at 5 x 10^5 cells/ml was seeded on 6 well plate in high glucose DMEM supplemented with 10% FBS and 1% penicillin-streptomycin in 37°C incubator, 98% humidified and 5% CO_2. After 15 days, Caco-2 cell was washed in PBS and replaced with 1% FBS antibiotic free DMEM[14]. Each well was treated with 10% condition media (CM), 20% sonicated cells (SON), heat killed cells or viable cells of three *Lactobacillus* spp. and stimulated with final concentration of 20 ug/ml lipopolysaccharide (LPS) from *Escherichia coli* (Sigma, USA) for 24 h. Collection of supernatants were centrifuged at 3,000 rpm, 4°C, 5 min and were used to detect TNF-α production by sandwich ELISA kit. The each samples experiment was duplicated.

The percentage of cell viability will be calculated by the following

% Cell viability = 100 × (1-(dead cells ÷ total cells).

4.2 The study of probiotic *Lactobacillus* strains in the suppression of TNF-α secretion in HepG-2 cells

HepG-2 cell was seeded at 1 x 10^5 cell/well on 24 well plate for 3 days.[26] The cell confluence at about 80% and each well was treated with 10% v/v conditioned media, 20 % v/v heated kill cells, sonicated cells or viable cells of three *Lactobacillus* spp. were stimulated with final concentration of 5 µg/ml lipopolysaccharide for 48 h. Collection of supernatants were centrifuged at 3,000 rpm, 4°C, 5 min and were detected TNF-α production by sandwich ELISA kit.

5. Detection of tumor necrosis factor (TNF-α) by sandwich ELISA kit

Detection of tumor necrosis factor (TNF-α) secretion in Caco-2 cell line and HepG-2 cell line[127] was performed by sandwich ELISA kit. Briefly, ELISA plates: 96 well plate: High binding (Nunc, USA) were coated with mouse anti-human TNF-α antibodies overnight. ELISA plates were washed three times with washing buffer (0.05% Tween 20 in 1x PBS) and non-specific binding were blocked with 1% BSA in PBS for 2 h. ELISA plates

were washed three times and samples supernatant from treated cell line were added. Recombinant human TNF-α was used a standard (R&D Systems, USA) and standard was diluted at the concentration of 7.8,15.625, 31.5, 62.5, 125, 250, 500, 1,000 pg/ml. Reagent diluent was used for blank and plates were incubated overnight. The plates were washed and biotinylated goat anti-human TNF-α detection antibodies was added and incubated for 2 h at room temperature and avoid direct contact with light and washed the plates before addition of streptavidin-horseradish peroxidase conjugate and incubated for 20 minute at room temperature. After that, ELISA plates were washed and Tetramethylbenzidine (R&D Systems, USA) was added and incubated for 20 minute and stop reaction by addition of H_2So_4. Absorbance was measured at 450 nm using BioTek® Synergy™ HT (Multi-Detection Microplate Reader, USA). ELISA plates were performed at 25°C. Results of TNF-α concentration was compared with standard curve and expressed as pg/ml.

The percentage of TNF-α inhibition will be calculated by the following

% TNF-α inhibition = 100 × ((observed ÷ baseline -1)

Where observed = secreted TNF-α of experiment (pg/ml) and baseline = secreted TNF-α of MRS bacterial culture medium (pg/ml).

6. Animal experiment

6.1 Induction of colitis

For colitis model, 24 wistar rat (*Rattus norvegicus*) with aging between 8-10 week and weighing 250-300 g were purchased from Namura Siam International. Housing of animal was controlled at temperature of 22 ± 5 °C, humidity: 55 ± 5%, 12 h: 12 h light/dark at Medical Center Animal Care Laboratory, Srinakharinwirot University, Thailand. The rats were adapted in environment for 1 week before experiment. Animal experiment was informed license no. COA/AE-002-2563 from Animal Ethics Committee of the Srinakharinwirot University. Animal was divided into 4 groups (6 rats per group) (Table 4 and Figure 11).[128, 129]

Table 4 Experimental design of colitis rats

Group No.	Group	
1	Negative control	standard diet + drinking water
2	Probiotic control	standard diet + viable cell of *L. paracasei* MSMC 39-1
3	Colitis control	standard diet + 3 % w/v Dextran sulfate sodium in drinking water
4	Probiotic test	standard diet + 3 % w/v Dextran sulfate sodium in drinking water + viable cell of MSMC39-1

* Probiotic was administered in probiotic control and probiotic test colitis for 14 days, Rat was administered oral gavage live MSMC39-1 $1x10^9$ CFU/ml/rat/day, standard diet was ad libitum. Dextran sulfate sodium was reagent able induced colitis pathogenesis.

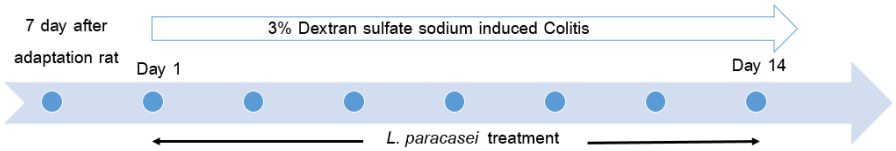

Figure 11 Time line experiment of colitis

6.1.1 Body weight and colon length detection in colitis rats

After the end experiment, all rats were weighed body weight and anesthetized with isoflurane, bood collection, removed colon for rinse and weighing, measurement colon length as centimeter unit for observation symtoms of the colitis.

6.1.2 Blood collection in colitis rat

After all rats were anesthetized and collected blood from cardiac puncture. Serum was separated from blood sample by centrifuged at 1500 g for 10 minute and kept at -20 °C for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) level detection.

6.1.3 Colon preparation

After 7 days, rat was anesthetized with isoflurane and sacrificed. Small and large intestine was removed and weighed. Colon tissue was removed, rinsed, and excised of tissue. The colon was homogenized with lysis buffer and centrifuge. The supernatant was collected for TNF-α detection. Measurement of colon length as centrimeter unit.

The pieces of colon tissue was fixed in 4% paraformaldehyde at 4°C for histology examination [130]Alcohol at 10%, 20%, 50%, 95% and 100% was used for colon tissue dehydration. The samples was embedded on paraffin.

6.1.4 Histology evaluation of colitis

Specimens of colitis in paraffin block were sectioned 5-7 μM using microtome. The sections were deparaffinized with xylene and rehydrated with 100%, 95% and 70% alcohol, respectively. The section slides were stained with hematoxylin & eosin (H&E). Histology examination of colitis was observed and scored according to Table 5.[130]

Table 5. Colitis score

Score	Characteristics
0	no ulceration and inflammation
1	no ulceration and local hyperemia
2	ulceration without hyperemia
3	ulceration and one site only of inflammation
4	two or more sites of ulceration and inflammation
5	ulceration extending more than 2 cm

6.1.5 Fecal collection of colitis rat

Stool of rats were collected before and after administration of *L. paracasei* MSMC39-1 in experiment of colitis rats for detection of gut microbiota.

6.2 Induction of hepatitis

For alcoholic hepatitis model, Wistar rats (*Rattus norvegicus*), 24 rats age 8-10 weeks weigh 250-300 g were purchased from Namura Siam International were used. Housing of animal was controlled at temperature of 22 ± 2 °C, humidity: 55 ± 5%, 12 h: 12 h light/dark [27, 131]. The rats were adapted in environment for 1 week before experiment. Animal experiment was informed license no. 9/2561 from Animal Ethics Committee of the Faculty of Medicine, Srinakharinwirot University. The experiment of hepatitis was divided into 4 groups (6 rat per group), (Table 6 and Figure 12).

Table 6 Experimental design of hepatitis rats

Group No.	Group	
1	Negative control	standard diet + distilled water
2	Probiotic control	standard diet + viable cell of *L. paracasei* MSMC 39-1
3	Hepatitis control	standard diet + 30% alcohol
4	Probiotic test	standard diet + 30 % alcohol + viable cells of *L. paracasei* MSMC 39-1 (*L. paracasei* was administered before induce hepatitis for 2 weeks and was treated continuing to 8 weeks)

Rat was administered oral gavage live MSMC39-1 $1x10^9$ CFU/ml/rat/day, standard diet was ad libitum.

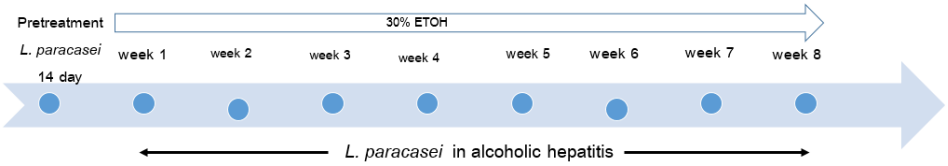

Figure 12 Time line experiment of hepatitis

6.2.1 Blood collection in hepatitis rat

Hepatitis rat was anesthetized with isoflurane and blood will be collected from cardiac puncture.[23] Serum was separated from blood sample by centrifuged at 1500 g for 10 minute. Aspartate aminotransferase (AST) and alanine aminotransferase [27] levels were detected in serum.

6.2.2 Liver preparation

After treatment, rats was anesthetized with isoflurane and sacrificed, right lobe of liver were removed and weighed.[23] The liver tissue was removed, rinsed, and excised of tissue. The right lobe of liver was homogenized with lysis buffer and centrifuge. The supernatant was collected for TNF-α detection.

The pieces of liver tissue was fixed in 4 % paraformaldehyde at 4 °C for histology examination. Alcohol at 10%, 20%, 50%, 95% and 100% was used for liver tissue dehydration. The samples were embedded on paraffin.

6.2.3 Histology evaluation of hepatitis rat

Specimen of hepatitis rats in paraffin block were sectioned at about 5-7 μM using microtome. The sections were deparaffinized with xylene and dehydrate with 100%, 95% and 70% alcohol, respectively. The section slides were stained with hematoxylin & eosin.

6.2.4 Fecal collection of hepatitis rat

Stool of rats were collected before and after administration of *L. paracasei* MSMC39-1 in experiment of alcoholic hepatitis for detection of gut microbiota.

7. Detection of tumor necrosis factor (TNF-α) in the colon and liver tissue of rat model by sandwich ELISA kit

Detection of Tumor necrosis factor (TNF-α) production in the supernatant of colon tissue of colitis rats and liver tissue of hepatitis rat model[112] by sandwich ELISA kit briefly, ELISA plate: 96 well plate: High binding (Nunc, USA) was coated with mouse anti-rat TNF-α antibodies overnight. The plate was washed three times with washing buffer (0.05% Tween 20 in 1x PBS) and non-specific binding was blocked with 1% BSA in PBS for 2 h. ELISA plate was washed three times and supernate of all rat was added. Recombinant rat TNF-α was used a standard (R&D Systems, USA) and standard was diluted at the concentration of 7.8,15.625, 31.5, 62.5, 125, 250, 500, 1,000 pg/ml. Reagent diluent was used for blank and plate was incubated overnight. The plate was washed and biotinylated goat anti-rat TNF-α detection antibodies was added and incubated for 2 h at room temperature and avoid direct contact with light and washed the plate before addition

of streptavidin-horseradish peroxidase conjugate and incubated for 20 minute at room temperature. After that, ELISA plate was washed and Tetramethylbenzidine (R&D Systems, USA) was added and incubated for 20 minute and stop reaction by addition of H_2So_4. Absorbance was measured at 450 nm using BioTek® Synergy™ HT (Multi-Detection Microplate Reader, USA). ELISA plate was performed at 25°C. Results of TNF-α concentration was compared with standard curve and expressed as pg/ml.

The percentage of TNF-α inhibition will be calculated by the following

% TNF-α inhibition = 100 × ((observed ÷ baseline -1)

8. Study of alteration of gut microbiota using Next generation sequencing

Stool collected from colitis and hepatitis rats were used for DNA extraction. DNA extraction using QIAamp DNA Stool Minikit (Qiagen)[132] was performed as manual briefly, fresh or frozen stool was weighed 180-220 mg in 2 ml microcentrifuge tube. InhibitEx buffer was added in each tube, heat at 70ºC for 5 min and vortex. The suspension was centrifuged, keep supernatant. Supernatant was transferred to new centrifuge tube and proteinase K, AL buffer were added and incubated at 70ºC for 10 min. Lysate was added with ethanol, vortex and add to new QIAamp spin column and centrifuged. AW1 buffer was added in the QIAamp spin column and centrifuged. AW2 buffer was added to QIAamp spin column, centrifuged and discarded filtrate tube. QIAamp spin column was transferred to 1.5 ml microcentrifuge tube and was added with ATE buffer and centrifuge for elute DNA. DNA concentration were measured by Nanodrop 2000 spectrophotometer (Thermo scientific, USA). The primers used in this study were as followed: forward primer:5'TCGTCGGCAGCGTCAGATGTGTATAAGA GACAGCCTACGGGNGGCWGCAG3'and reverse primer:5'GTCTCGTGGGCTCGGA GATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC3'. Two variable regions of 16S rDNA (V3 and V4) of 16S Metagenome were sequenced by illumina Miseq sequencer (Illumina, USA) [133].

9. Statistical analysis

Statistical significance or differences were evaluated by Graphpad Prism version 8.0.2, one-tailed distribution of Student t-test. A ρ-value ≤ 0.05 was considered statistically significantly.

For animal study was used one way ANOVA with comparing each group with control group express as mean $\pm$ SD

CHAPTER 4
RESULT

1. The effect of *Lactobacillus* strains on the TNF-α secretion in Caco-2 cells

Previous study showed that *L. paracasei* MSMC 39-1, *L. casei* MSMC 39-3 and *Weissella confusa* MSMC57-1 inhibited TNF-α production in THP-1 monocytic cell line [127]. In this study, the effects of *L. paracasei* MSMC 39-1, *L. casei* MSMC 39-3 and *Weissella confusa* MSMC57-1 on TNF-α secretion were examined in Caco-2 cells activated with lipopolysaccharides.

Four various antigens including probiotic culture supernatant, viable cells, heat killed cells and sonicated cells were prepared from the strains of *Lactobacillus* spp. TNF-α secretion in Caco-2 cells activated with lipopolysaccharides for 24 h was examined in the presence of four antigens of each strain at final concentration of 10^9 CFU/ml. The results indicated that probiotic culture supernatant of *L. paracasei* MSMC 39-1, *L. casei* MSMC 39-3 and *Weissella confusa* MSMC57-1 slightly suppressed TNF-α secretion in Caco-2 cells activated with lipopolysaccharides as shown in Table 7 and Figure 13.

Viable cells of *L. paracasei* MSMC 39-1, *L. casei* MSMC 39-3 and *Weissella confusa* MSMC57-1 exhibited significantly strong suppression of TNF-α secretion in Caco-2 cells activated with lipopolysaccharides as shown in Table 8 and Figure 14 (**$\rho < 0.01$). In addition, a significantly strong suppression of TNF-α secretion was also observed in Heat-killed cells of *L. paracasei* MSMC 39-1 and *L. casei* MSMC 39-3 as shown in Figure 15 (*$\rho < 0.05$). TNF-α secretion in Caco-2 cells activated with lipopolysaccharides was moderately suppressed by Heat-killed cell of *Weissella confusa* MSMC57-1 as shown in Table 9 and Figure 15. Sonicated cells of *L. casei* MSMC 39-3 and *Weissella confusa* MSMC57-1 exhibited strong and slight suppression of TNF-α secretion in Caco-2 cells activated with lipopolysaccharides when compared with the MRS control as shown in Table 10 and Figure 16 (*$\rho < 0.05$). On the other hand, sonicated cells of *L. paracasei* MSMC 39-1 had no effect on TNF-α secretion in Caco-2 cells

activated with lipopolysaccharides when compared with the MRS control as shown in Figure 16.

Table 7 Effect of probiotic culture supernatant of *Lactobacillus* strains on the TNF-α secretion in Caco-2 cells activated lipopolysaccharides. MRS control; SD, standard deviation.

Probiotic culture supernatant + LPS	TNF- α (ρg/ml)	SD	% inhibition
MRS + LPS	1160	28.28	
L. paracasei MSMC 39-1	1123	4.29	3.19
L. casei MSMC39-3	1120	14.14	3.45
Weissella confusa MSMC57-1	1135	28.28	2.15

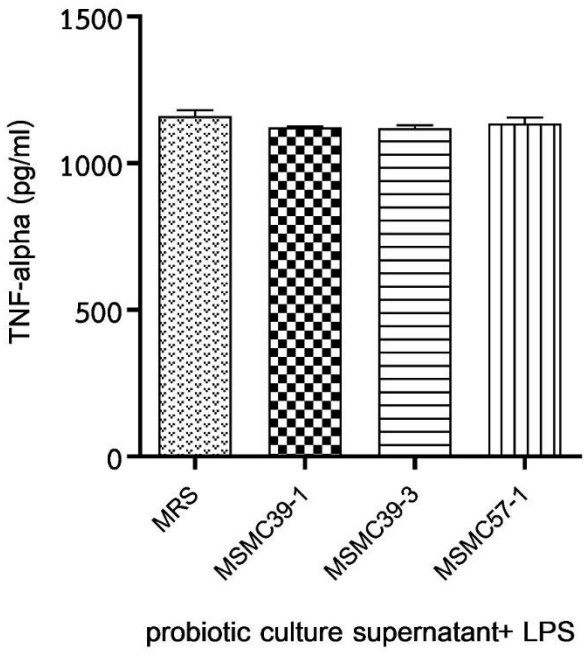

Figure 13 Effect of probiotic culture supernatant of *Lactobacillus* strains on the TNF-α secretion in Caco-2 cells activated lipopolysaccharides.

Experiments were performed duplicate and three times, MRS control. Statistical analysis was used one-tailed Student t-test distribution. Error bars showed standard deviations, * ($\rho < 0.05$).

Table 8 Effect of viable cells cell *Lactobacillus* strains on the TNF-α secretion in Caco-2 cells activated lipopolysaccharides. MRS control; SD, standard deviation.

Viable cells + LPS	TNF-α (ρg/ml)	SD	% inhibition
MRS + LPS	1185	21.21	
L. paracasei MSMC 39-1	977.50	36.86	17.51
L. casei MSMC39-3	952.50	42.72	19.62
Weissella confusa MSMC57-1	962.50	49.24	18.78

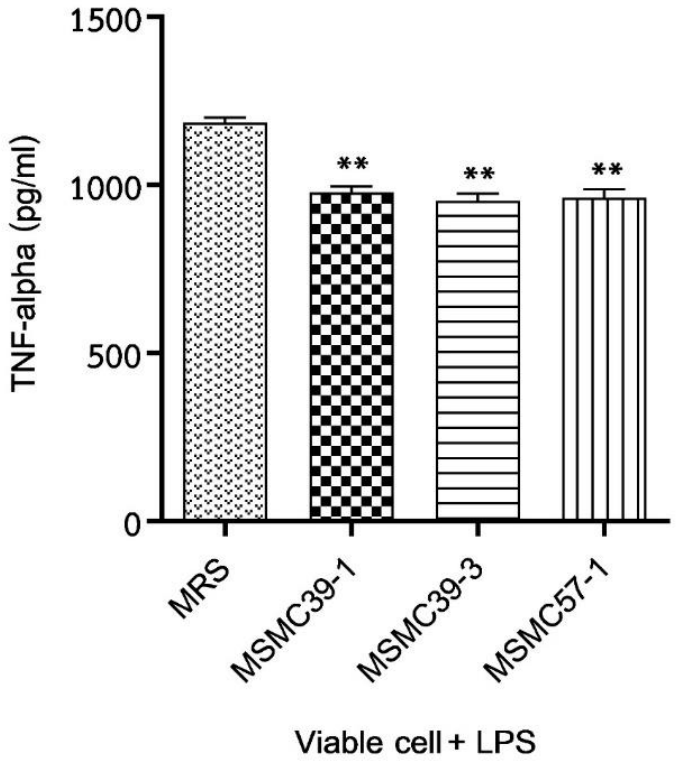

Figure 14 Effect of viable cells cell *Lactobacillus* strains on the TNF-α secretion in Caco-2 cells activated lipopolysaccharides.

Experiments were performed duplicate and three times, MRS control. Statistical analysis was used one-tailed Student t-test distribution. Error bars showed standard deviations, * ($\rho < 0.05$); ** ($\rho < 0.01$).

Table 9 Effect of heat-killed cell *Lactobacillus* strains on the TNF-α secretion in Caco-2 cells activated lipopolysaccharides. MRS control; SD, standard deviation.

Heat killed cells + LPS	TNF-α (ρg/ml)	SD	% inhibition
MRS + LPS	657.4	84.43	
L. paracasei MSMC 39-1	408.6	79.02	37.85
L. casei MSMC39-3	393.50	68.87	40.14
Weissella confusa MSMC57-1	444.50	66.26	32.38

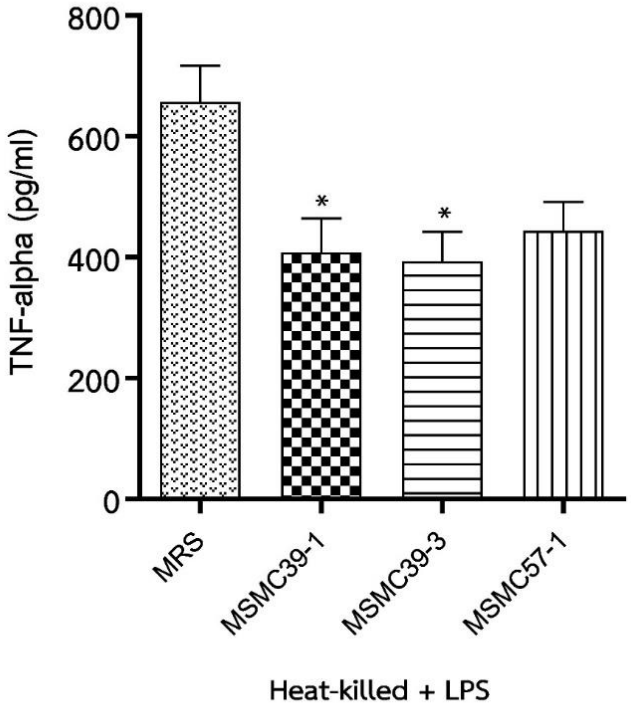

Figure 15 Effect of heat-killed cell *Lactobacillus* strains on the TNF-α secretion in Caco-2 cells activated lipopolysaccharides.

Experiments were performed duplicate and three times, MRS control. Statistical analysis was used one-tailed Student t-test distribution. Error bars showed standard deviations, * ($\rho < 0.05$).

Table 10 Effect of sonicated cell *Lactobacillus* strains on the TNF-α secretion in Caco-2 cells activated lipopolysaccharides. MRS control; SD, standard deviation.

Sonicated cells + LPS	TNF-α (ρg/ml)	SD	% inhibition
MRS + LPS	1,120	28.28	
L. paracasei MSMC 39-1	1,131	42.43	0.98
L. casei MSMC39-3	1,044	28.28	6.78
Weissella confusa MSMC57-1	1,025	5.66	8.48

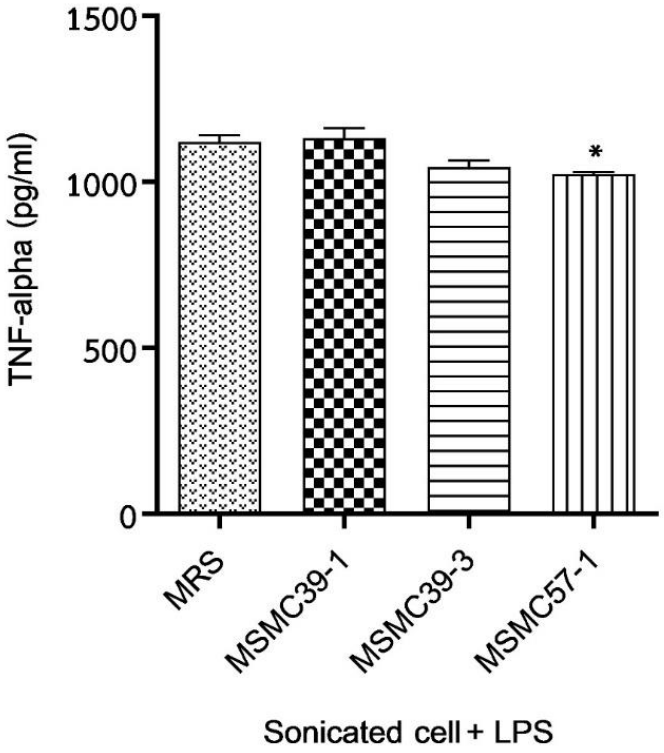

Figure 16 Effect of sonicated cell *Lactobacillus* strains on the TNF-α secretion in Caco-2 cells activated lipopolysaccharides.

Experiments were performed duplicate and three times, MRS control. Statistical analysis was used one-tailed Student t-test distribution. Error bars showed standard deviations, * ($\rho < 0.05$).

2. The effect of *Lactobacillus* strains in the suppression of TNF-α secretion in HepG-2 cells

Previous study showed that *L. paracasei* MSMC 39-1, *L. casei* MSMC 39-3 and *Weissella confusa* MSMC57-1 inhibited TNF-α production in THP-1 monocytic cell line [127]. In this study, the effects of *L. paracasei* MSMC 39-1, *L. casei* MSMC 39-3 and

Weissella confusa MSMC57-1 on TNF-α secretion were examined in HepG-2 cells activated with lipopolysaccharides.

Four various antigens including probiotic culture supernatant, viable cells, heat killed cells and sonicated cells were prepared from the strains of *Lactobacillus* spp. These antigens from each strain were used to test the suppression of TNF-α secretion in HepG-2 cells activated with lipopolysaccharides. Probiotic culture supernatant of *L. paracasei* MSMC 39-1 and *L.casei* MSMC39-3 had slight effects on TNF-α secretion as shown in Table 11 and Figure 17. However, probiotic culture supernatant of *Weissella confusa* MSMC57-1 had no effect on TNF-α secretion as shown in Table 11 and Figure 17.

A significant strong suppression of TNF-α secretion was observed in viable cells of *L. paracasei* MSMC 39-1 when compared with MRS control (***$\rho < 0.001$) as shown in Table 12 and Figure 18. Viable cells of *L.casei* MSMC39-3 and *Weissella confusa* MSMC57-1 exhibited moderate suppression of TNF-α secretion in HepG-2 cells activated with lipopolysaccharides as shown in Table 12 and Figure 18.

Heat killed cell of *L. paracasei* MSMC 39-1 significantly suppressed TNF-α secretion in HepG-2 cells when compared to MRS controls (*$\rho < 0.05$) as shown in Table 13 and Figure 19. Heat killed cell of *L. casei* MSMC39-3 slightly inhibited TNF-α secretion in HepG-2 cells, and heat killed cell of *Weissella confusa* MSMC57-1 had no effect on TNF-α secretion in HepG-2 cells as shown in Table 13 and Figure 19.

Sonicated cell of *Weissella confusa* MSMC57-1 significantly suppressed TNF -α secretion in HepG-2 cells (*$\rho < 0.05$) as shown in Table 14 and Figure 20. Sonicated cell of *L. paracasei* MSMC 39-1 and *L. casei* MSMC39-3 slightly suppressed TNF -α secretion in HepG-2 cells as shown in Table 14 and Figure 20.

Table 11 Effect of probiotic culture supernatant *Lactobacillus* strains on the TNF-α secretion in HepG-2 cells activated lipopolysaccharides. MRS control; SD, standard deviation.

Probiotic culture supernatant + LPS	TNF-α (ρg/ml)	SD	% inhibition
MRS + LPS	527.5	3.54	
L. paracasei MSMC 39-1	515.0	7.07	2.37
L. casei MSMC39-3	520.0	14.14	1.42
Weissella confusa MSMC57-1	560.0	28.28	1.44

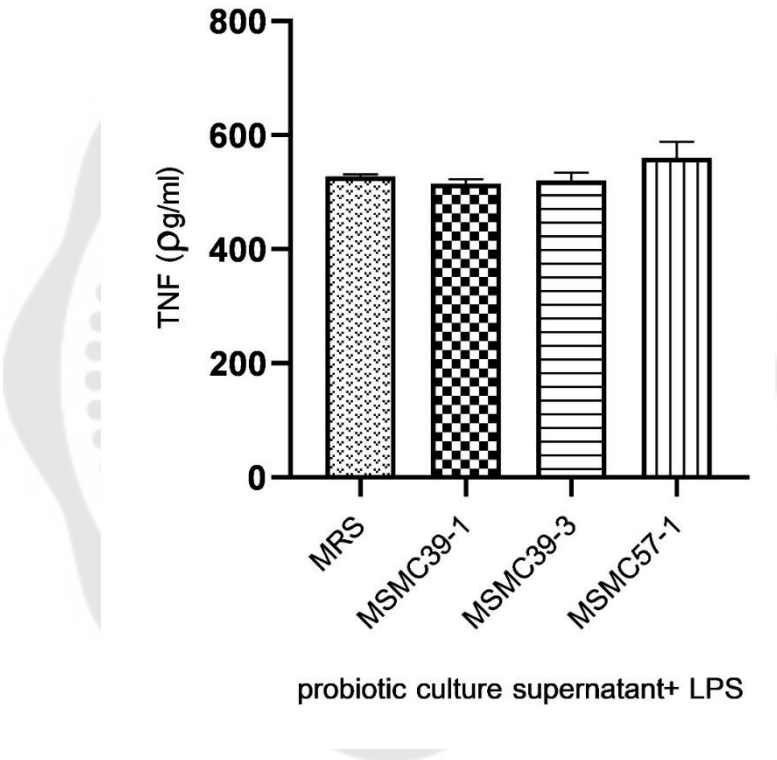

Figure 17 Effect of probiotic culture supernatant *Lactobacillus* strains on the TNF-α secretion in HepG-2 cells activated lipopolysaccharides.

Experiments were performed duplicate and three times, MRS control. Statistical analysis was used one-tailed Student t-test distribution. Error bars showed standard deviations, * ($\rho < 0.05$).

Table 12 Effect of viable cell *Lactobacillus* strains on the TNF-α secretion in HepG-2 cells activated lipopolysaccharides. MRS control; SD, standard deviation.

Viable cells + LPS	TNF-α (ρg/ml)	SD	% inhibition
MRS + LPS	535.0	1.41	
L. paracasei MSMC 39-1	249.0	8.48	53.45
L. casei MSMC39-3	410.0	11.31	23.36
Weissella confusa MSMC57-1	412.5	43.13	22.89

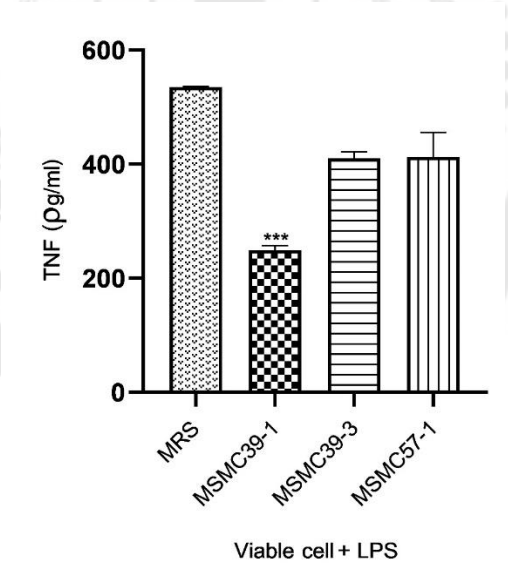

Figure 18 Effect of viable cell *Lactobacillus* strains on the TNF-α secretion in HepG-2 cells activated lipopolysaccharides.

Experiments were performed duplicate and three times, MRS control. Statistical analysis was used one-tailed Student t-test distribution. Error bars showed standard deviations, * ($\rho < 0.05$); ** ($\rho < 0.01$); *** ($\rho < 0.001$).

Table 13 Effect of heat killed cell *Lactobacillus* strains on the TNF-α secretion in HepG-2 cells activated lipopolysaccharides. MRS control; SD, standard deviation.

Heat killed cells + LPS	TNF-α (ρg/ml)	SD	% inhibition
MRS + LPS	535.0	1.41	
L. paracasei MSMC 39-1	303.5	68.59	43.27
L. casei MSMC39-3	435.0	43.84	18.69
Weissella confusa MSMC57-1	796.5	119.50	48.88

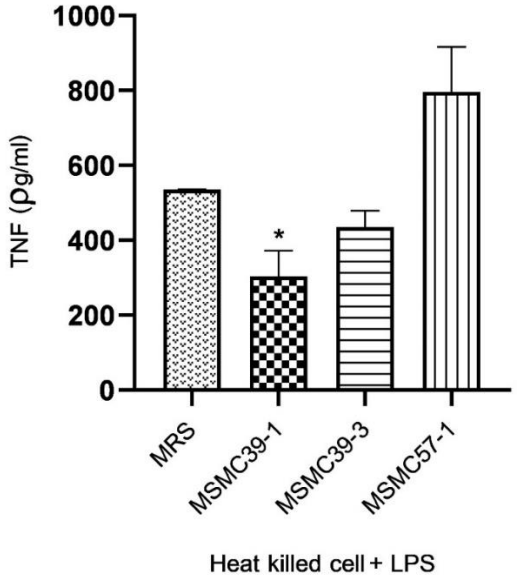

Figure 19 Effect of heat killed cell *Lactobacillus* strains on the TNF-α secretion in HepG-2 cells activated lipopolysaccharides.

Experiments were performed duplicate and three times, MRS control. Statistical analysis was used one-tailed Student t-test distribution. Error bars showed standard deviations, * ($\rho < 0.05$).

Table 14 Effect of sonicated cells *Lactobacillus* strains on the TNF-α secretion in HepG-2 cells activated lipopolysaccharides. MRS control; SD, standard deviation.

Sonicated cells + LPS	TNF-α (ρg/ml)	SD	% inhibition
MRS + LPS	535.0	1.41	
L. paracasei MSMC 39-1	527.5	16.26	1.40
L. casei MSMC39-3	500.5	81.32	6.44
Weissella confusa MSMC57-1	346.5	44.55	35.23

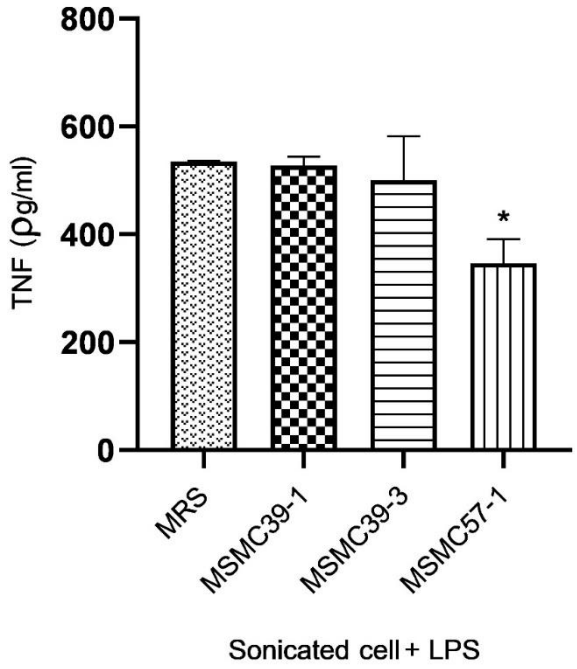

Figure 20 Effect of sonicated cell *Lactobacillus* strains on the TNF-α secretion in HepG-2 cells activated lipopolysaccharides.

Experiments were performed duplicate and three times, MRS control. Statistical analysis was used One-tailed Student t-test distribution. Error bars indicated standard deviations, * ($\rho < 0.05$); ** ($\rho < 0.01$); *** ($\rho < 0.001$).

3. Effect of *Lactobacillus paracasei* MSMC39-1 on body weight and colon length in colitis rats

Due to the strongest effect on TNF-α secretion in Caco-2 cells and HepG-2 cells, *L. paracasei* MSMC 39-1 was selected in animal model. In the colitis experiment, body weight loss, diarrhea with bleeding and colon shorten were observed in colitis rats with oral gavage with 3% DSS. However, body weight loss was observed in these rats when

compared to normal control, probiotic control, and probiotic test group as shown in Table 15 and Figures 21-22. There was no difference in body weight, and colon length of rats in negative control, probiotic control, and probiotic test group when compared to the control group as shown in Table 15. Thus, DSS induced colitis caused weight loss, colon length and diarrhea with bleeding.

Table 15 Effect of *L. paracasei* MSMC39-1 on body weight increase and colon length

Parameters	Group			
	Negative control	Probiotic control	Colitis control	Probiotic test
Body weight increase (g)	125 ± 9.98	123 ± 23.91	93 ± 6.80	107 ± 11.03
Colon length (cm)	16 ± 1.73	17 ± 2.71	14 ± 1.54	14.8 ± 0.67

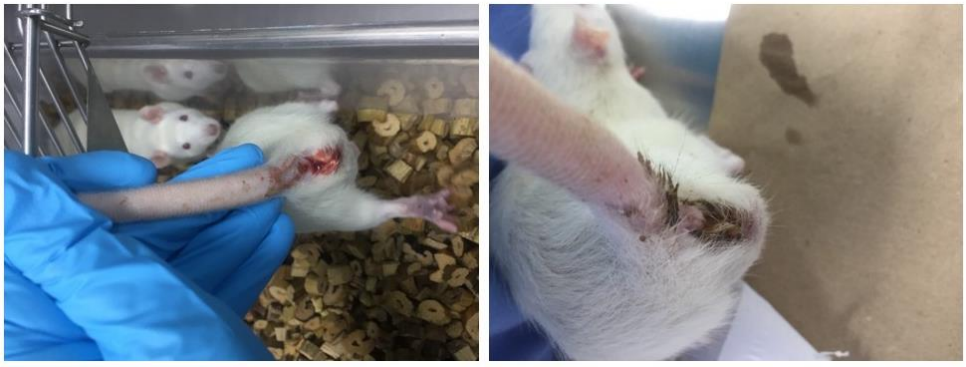

Figure 21 Symtoms of colitis rats

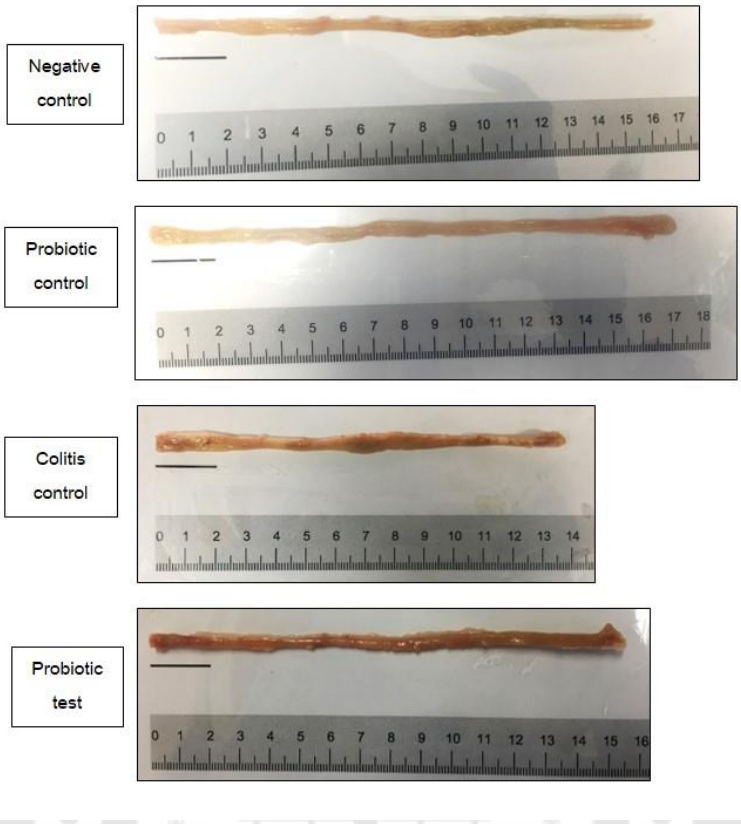

Figure 22 Colon length after *L. paracasei* MSMC39-1 treatment in colitis rats.

4. Effect of *Lactobacillus paracasei* MSMC39-1 on liver enzyme activity in colitis rat

A high level of serum aspartate aminotransferase (AST) was observed in colitis control group (Figure 23). Aspartate aminotransferase level significantly reduced in probiotic *L. paracasei* treatment colitis rat ($*\rho < 0.05$) (Figure 23) when compared to colitis control group. Alanine aminotransferase level in serum of colitis rat was high but ALT level was reduced when treated with *L. paracasei* (Figure 24).

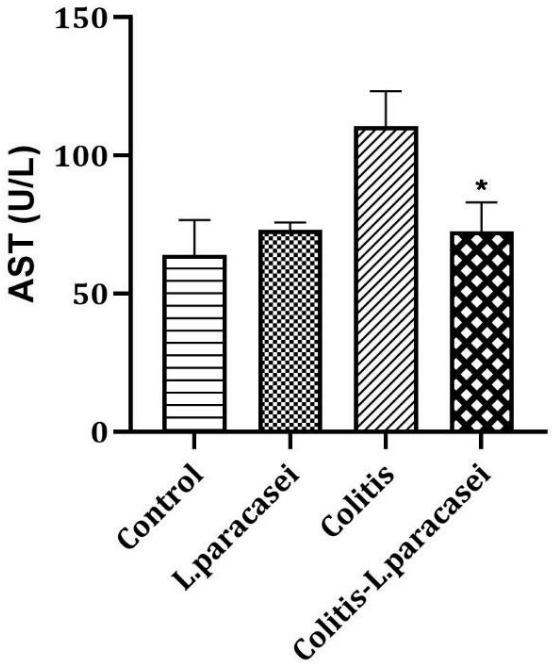

Figure 23 Effect of *L. paracasei* MSMC 39-1 on aspartate aminotransferase in DSS-induced colitis rats

Error bar was shown standard deviation. Statistical analysis used One way ANOVA and *$\rho < 0.05$** ($\rho < 0.01$); *** ($\rho < 0.001$); significant differences from the control (n =6 rats).

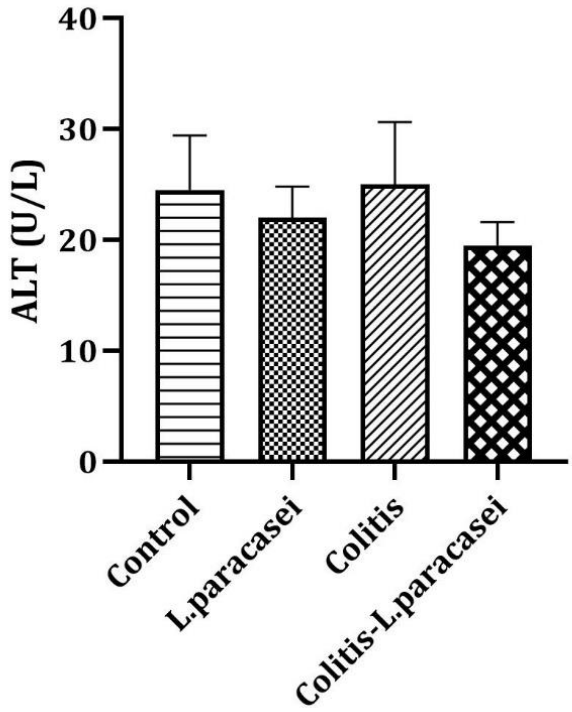

Figure 24 Effect of *L. paracasei* MSMC 39-1 on alanine aminotransferase in DSS-induced colitis rats.

Error bar was shown standard deviation. Statistical analysis used One way ANOVA and *$\rho < 0.05$** ($\rho < 0.01$); *** ($\rho < 0.001$); significant differences from the control (n =6 rats).

5. Effect of *Lactobacillus paracasei* MSMC39-1 on TNF-α secretion in colon tissue of DSS induced colitis rat

High level of TNF-α secretion was observed in colitis rats administered with 3% DSS (Figure 25). TNF-α levels of colon tissues from rats in probiotic *L. paracasei* treatment group were significantly lower that colitis control group (***$\rho < 0.001$) (Figure 25). The similar level of TNF-α was observed in colon tissues of normal control and probiotic control groups (Figure 25). Thus *L. paracasei* improved cotitis by inhibit TNF-α secretion in colon tissue of colitis rats.

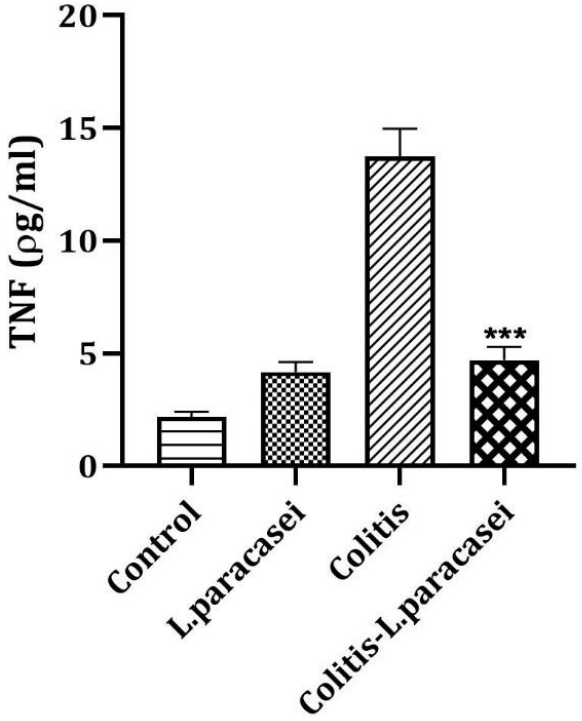

Figure 25 Effect of *L. paracasei* MSMC 39-1 on TNF-α secretion colon tissue in colitis rats.

Error bar was shown standard deviation. Statistical analysis used One way ANOVA and *$\rho < 0.05$** ($\rho < 0.01$); *** ($\rho < 0.001$); significant differences from the control (n =6 rats).

6. Effect of *Lactobacillus paracasei* MSMC39-1 on TNF-α secretion in liver tissue of DSS induced colitis rat

A high level of TNF-α secretion was observed in colitis rats administred with 3% DSS (Figure 26). TNF-α secretion in colitis rats in probiotic *L. paracasei* treatment group was significantly suppressed when compared to the colitis control group (***$\rho < 0.001$)

(Figure 26). The level of TNF-α in negative control and probiotic control group was similar in liver tissue (Figure 26).

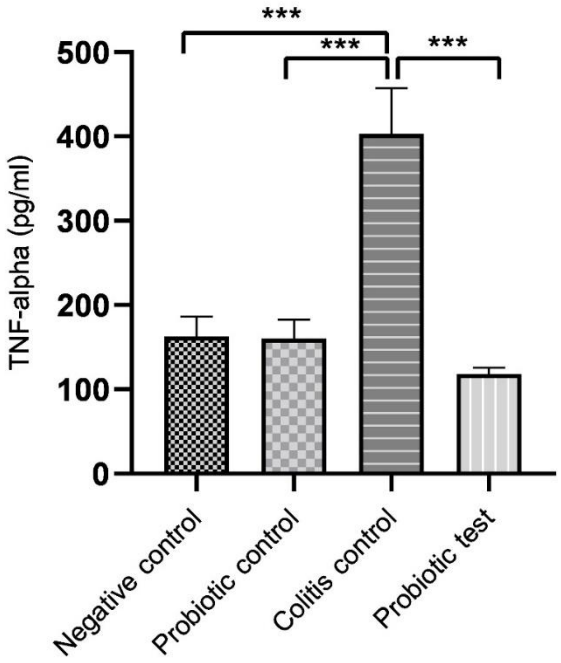

Figure 26 Effect of *L. paracasei* MSMC 39-1 on TNF-α secretion in liver tissue of DSS-induced colitis rats.

Experiments were performed duplicate and three times, MRS control. Statistical analysis was used One way ANOVA. Error bars indicated standard deviations, *($\rho < 0.05$; ** ($\rho < 0.01$); *** ($\rho < 0.001$).

7. Effect of *Lactobacillus paracasei* MSMC39-1 on colon tissue histology in DSS-induced colitis rat

Effect of probiotic *L. paracasei* administeration in colitis rats and histopathology of colon tissue was examined by haematoxylin and eosin staining. Colon tissue of normal control and probiotic control with colitis score were 0-1. The size of colon was regular with no ulceration and no inflammatory cells as shown in Figure 27-28. For colitis group, there

were two and three point of ulceration, numerous inflammatory cells infiltrated in the cell (red arrow, Figure 28), crypt absces, and crypt depth were decreased and colitis score was about 3 shown in Figure 27-29. Interestingly, colon tissue of colitis rats were significantly improved by viable cell probiotic *L. paracasei* MSMC39-1. There were few inflammatory cells in the cell, no ulceration, increased in crypt depth, and colitis score was about 1 as shown in Figures 27-29. Normal control probiotic control and probiotic test showed that crypt depth not different as shown in Figures 29.

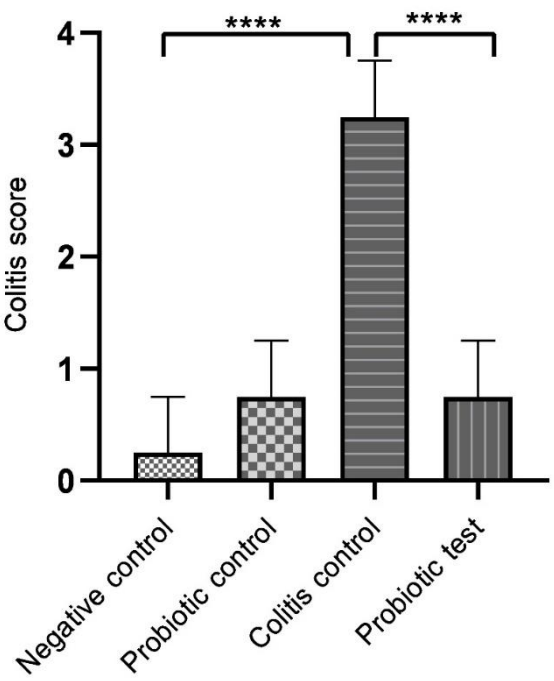

Figure 27 Colitis score of *L. paracasei* treatment

Experiments were performed n=6 rats, negative control. Statistical analysis was used One way ANOVA. Error bars indicated standard deviations, * ($\rho < 0.05$); ** ($\rho < 0.01$); *** ($\rho < 0.001$), **** ($\rho < 0.0001$).

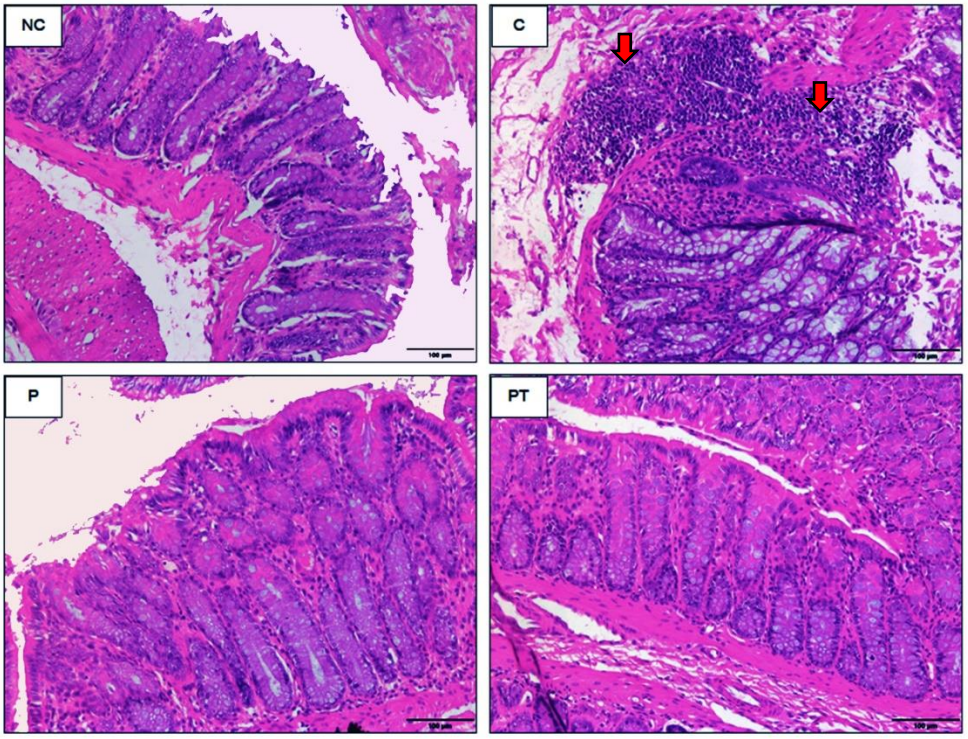

Figure 28 Colon histology of *L. paracasei* treatment in colitis rats.

Normal control (NC) and probiotic control (P) showed normal colon. Colitis (C) indicated intense inflammatory cell and improved by probiotic *L. paracasei* MSMC39-1 treatment (PT). 200x magnification and scale bar 100 μm.

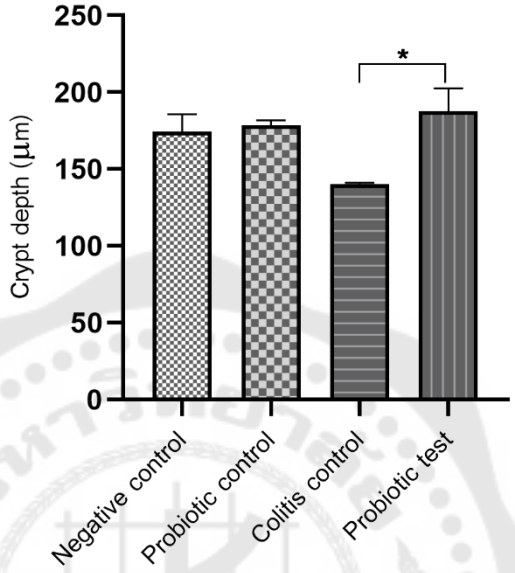

Figure 29 Histology of colon showing crypt depth in hemotoxylin and eosin staining in colitis rats

8. Effect of *Lactobacillus paracasei* MSMC39-1 on liver tissue histology in DSS induced-colitis rat

Effect of probiotic *L. paracasei* administeration in colitis rats and histopathology of liver tissue was examined by Haematoxylin and eosin staining.

The central vein was the center of hepatic cord which, was hepatocyte arrangement. Liver tissue of normal control and probiotic control showed the normal size of hepatocyte with no edema, no fat droplet and regularity hepatocyte arrangement as shown in Figure 30. For colitis group, a lot of fat foam were found in cytoplasm of the hepatocyte. Hepatocyte were big, swollen, and invisible to hepatic cord arrangement. In addition, hepatocyte was covered with some inflammatory cells. Interestingly, liver tissue of colitis rats was improved by viable cell probiotic *L. paracasei*. There were normal hepatic cord arrangement and few fat droplets in the hepatocyte as shown in Figure 30.

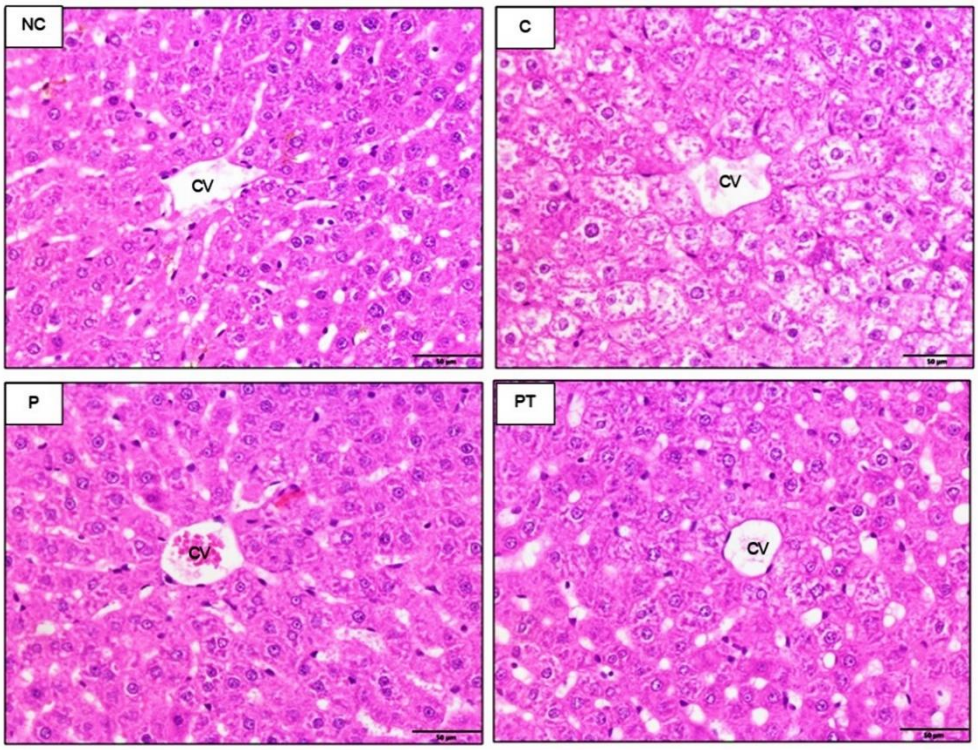

Figure 30 Liver histology of *L. paracasei* treatment in colitis rats.

Normal control (NC) and probiotic control (P) showed normal colon. Colitis (C) indicated intense inflammatory cell and improved by probiotic *L. paracasei* MSMC39-1 treatment (PT). 400x magnification scale bar 50 µm.

9. Effect of *Lactobacillus paracasei* MSMC39-1 on gut microbiota alteration in DSS-induced colitis rat

The next generation sequencing of colitis rats group showed an increase in Fermicutes as shown in Figure 31. After *L. paracasei* MSMC39-1 treatment in DSS induced colitis rats, the number of phylum Fermicutes slightly decreased whereas the number of Bacteroidetes increased when compared to colitis control as shown in Figure. 31. Mixure of good and bad microbiota happens due to a diarrhea which is colitis symtom. Heatmap showed an increase in genus *Lactobacillus* spp. in *L. paracasei* MSMC39-1 administeration in colitis rats when compared to colitis control as shown in Figure 32. In

colitis control group, the number of genus *Clostridium* pathogen or bad microbiota was higher than that of probiotic *L. paracasei* MSMC39-1 test as shown in Figure 32.

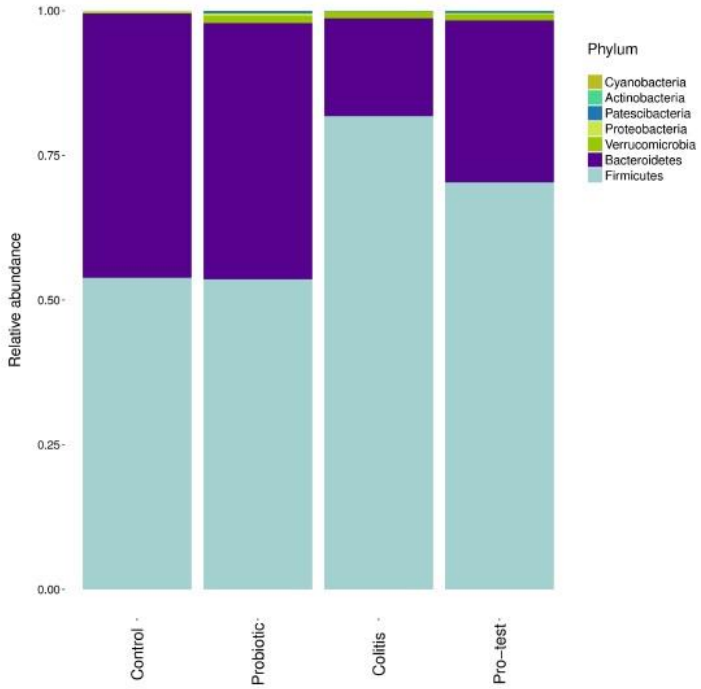

Figure 31 Effect of *L. paracasei* MSMC39-1 on gut microbiota alteration in colitis rats.

Statistical analysis used One way ANOVA and *ρ < 0.05; significant differences from the control (n =6 rats).

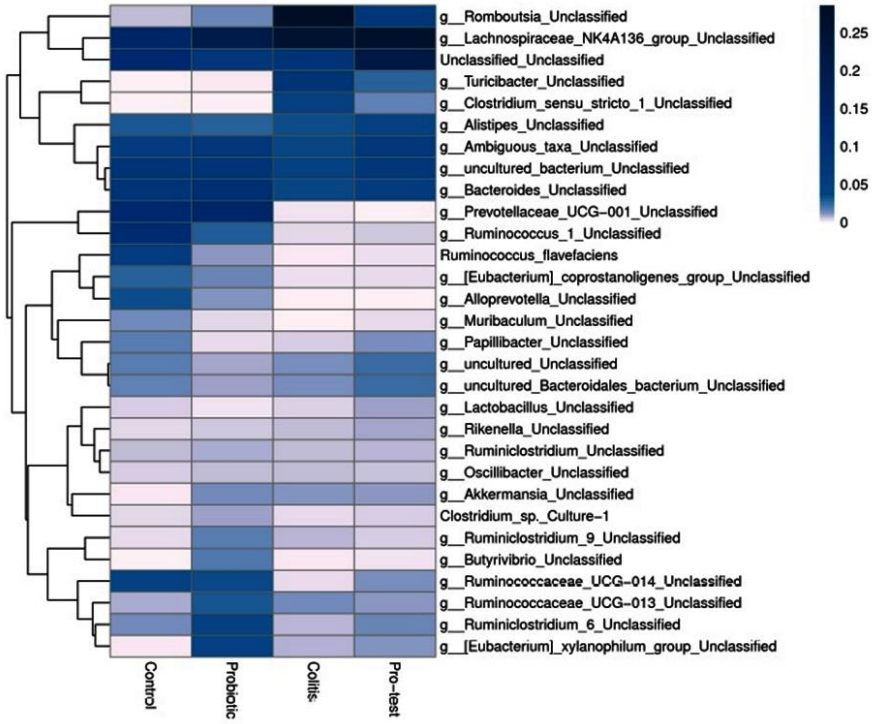

Figure 32 Heatmap of *L. paracasei* MSMC39-1 on gut microbiota alteration in colitis rats.

Statistical analysis used One way ANOVA and *ρ < 0.05; significant differences from the control (n =6 rats).

10. Effect of alcohol and *L. paracasei* MSMC39-1 on body weight and liver weight

Liver weight and liver-to-body weight ratio were increased in hepatitis rats with oral gavage with alcohol for 8 weeks. However, body weight loss was observed in these rats when compared to normal control, probiotic, and probiotic treatment group as shown in Table 16. There were no differences in body weight, liver weight and liver-to-body weight ratio of rats in normal, probiotic, and probiotic treatment group when compared to the control group as shown in Table 16. Thus alcohol induced hepatitis caused weight loss, liver edema, and increase in liver weigh.

Table 16 Effect of *L. paracasei* MSMC39-1 on body weight and liver weight

Parameters	Group			
	Normal	Probiotic	Alcohol	Probiotic treatment
Body weight (g)	486.4±6.93	462.5±25.14	403.1±10.39*	479.3±16.5[a]
Liver weight (g)	15.03±1.41	14.27± 0.43	15.83±4.86	15.1±1.28
Liver to body weight ratio (%)	3.08±0.25	3.09±0.08	3.94±1.3	3.15±0.05

The data are shown as mean ± SD. Statistical analysis used One way ANOVA. *ρ< 0.05 compared with the control groups; [#]ρ <0.05 compared with the probiotic treatment group; [a] ρ <0.05 compared with the alcohol group.

11. Effect of *Lactobacillus paracasei* MSMC39-1 on liver enzyme activity in alcohol-induced hepatitis rat

High level of serum aspartate aminotransferase (AST) was observed in rats in alcohol group for 8 weeks (Figure 33). Aspartate aminotransferase level significantly reduced in probiotic *L. paracasei* treatment hepatitis rat (*ρ < 0.05) (Figure 33) when compared to alcohol group. Alanine aminotransferase level [(25)] in serum of alcohol rat was high but ALT level was reduced when treatment with *L. paracasei* (Figure 34). Alanine aminotransferase level in the control and *L. paracasei* group were lower than that of alcohol group (Figure 34).

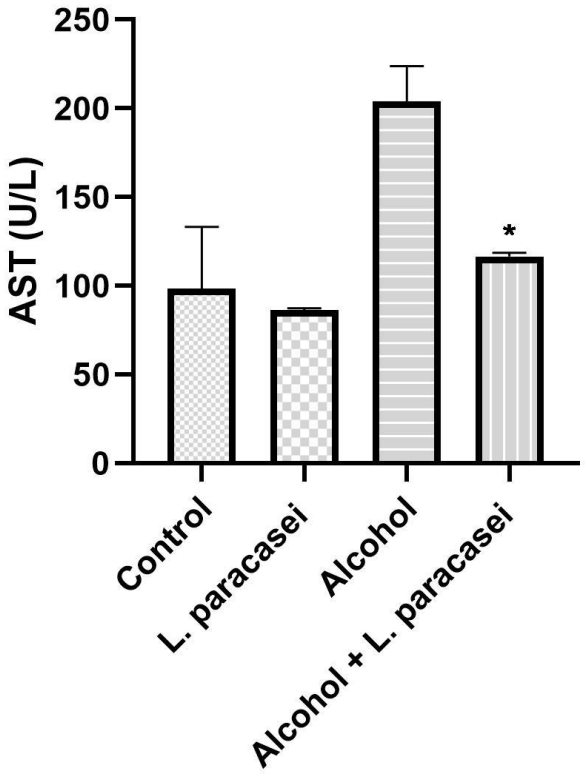

Figure 33 Effect of *L. paracasei* MSMC 39-1 on aspartate aminotransferase in hepatitis rats.

Error bar was shown standard deviation. Statistical analysis used One way ANOVA and *$\rho < 0.05$** ($\rho < 0.01$); *** ($\rho < 0.001$); significant differences from the control (n =6 rats).

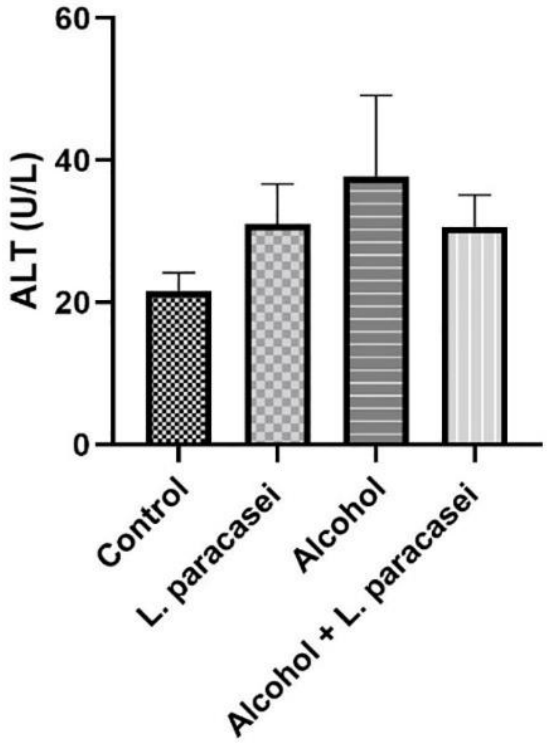

Figure 34 Effect of *L. paracasei* MSMC 39-1 on alanine aminotransferase in hepatitis rats.

Error bar was shown standard deviation. Statistical analysis used One way ANOVA and *$\rho < 0.05$** ($\rho < 0.01$); *** ($\rho < 0.001$); significant differences from the control (n =6 rats).

12. Effect of *Lactobacillus paracasei* MSMC39-1 on TNF- -α in liver tissue of alcohol-induced hepatitis rat

High TNF-α secretion was observed in hepatic tissue of hepatitis rats administered with 30% alcohol for 8 weeks (Figure 35). TNF-α levels in hepatic tissue of rat in probiotic *Lactobacillus paracasei* treatment group were lower than alcohol control

group (*ρ < 0.05) (Figure 35). Similar levels of TNF-α in liver tissues were observed in normal control and probiotic control group (Figure 35).

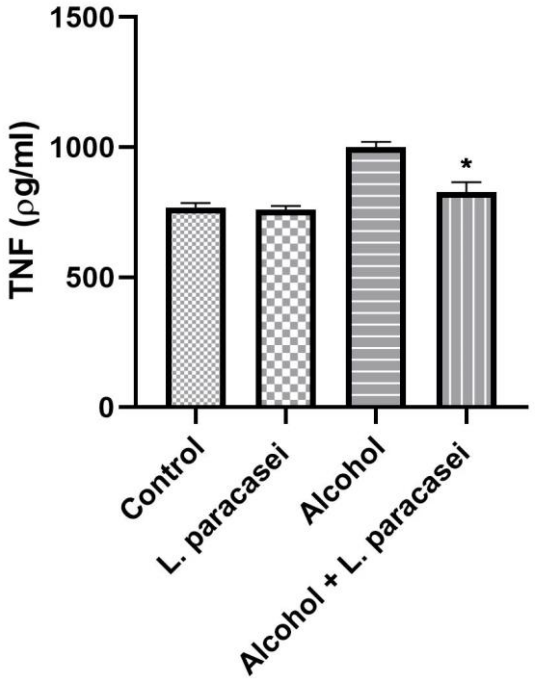

Figure 35 Effect of *L. paracasei* MSMC39-1 suppress TNF-α secretion in liver tissue of hepatitis rat.

Error bar was shown standard deviation. Statistical analysis used One way ANOVA and *ρ < 0.05; significant differences from the control (n =6 rats).

13. Effect of *Lactobacillus paracasei* MSMC39-1 on colon tissue histology in alcohol-induced hepatitis rat

Histopathology of colon tissues were examined by haematoxylin & eosin staining. Normal intestinal architectures of colon tissues were observed in normal and probiotic control groups as shown in Figure 36. Crypt edema and infiltration of inflammatory cells were found in alcoholic hepatitis colon tissues of rat administered with 30% alcohol for 8

weeks. Interestingly, probiotic *L. paracasei* MSMC39-1 treatment improved colon tissues of hepatitis rats with slight crypt edema and inflammatory cells as shown in Figure 36.

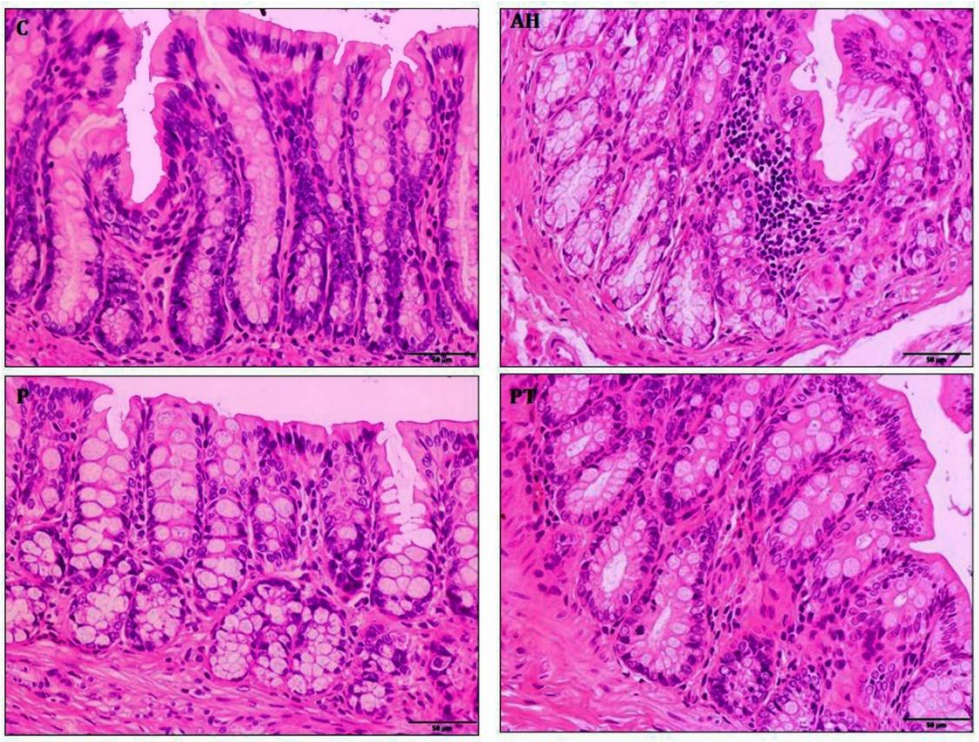

Figure 36 Histology of colon tissue was an estimate by hematoxylin and eosin staining in hepatitis rats.

Normal control (C) and probiotic control (P) showed normal crypt. Alcoholic hepatitis (AH) indicated intense inflammatory cell, crypt edema and improved by probiotic *L. paracasei* MSMC39-1 treatment (PT). 400x magnification scale bar 50 μm.

14. Effect of *Lactobacillus paracasei* MSMC39-1 on liver tissue histology in alcohol-induced hepatitis rat

Effect of 1 x 10^{9} CFU/ml viable cells of probiotic *L. paracasei* MSMC39-1 feeding in alcoholic hepatitis rats for 8 weeks and histopathology of liver tissue was examined by Haematoxylin and eosin staining. The central vein was center of hepatic cord which, was

hepatocyte arrangement. Liver tissue of normal control and probiotic control showed normal size of hepatocyte with no edema, no foam fat in the cells and regularity hepatocyte arrangement as shown in Figure 37. Administration of 30% alcohol for 8 weeks in alcoholic hepatitis rats showed a lot of fat foam in cytoplasm of hepatocyte, big and swollen hepatocyte, and invisible hepatic cord arrangement. Hepatocyte was covered with few inflammatory cells. Interestingly, liver tissues of hepatitis rats were improved by probiotic *L. paracasei*. There were normal hepatic cord arrangement and slight fat foam in the hepatocyte as shown in Figure 37.

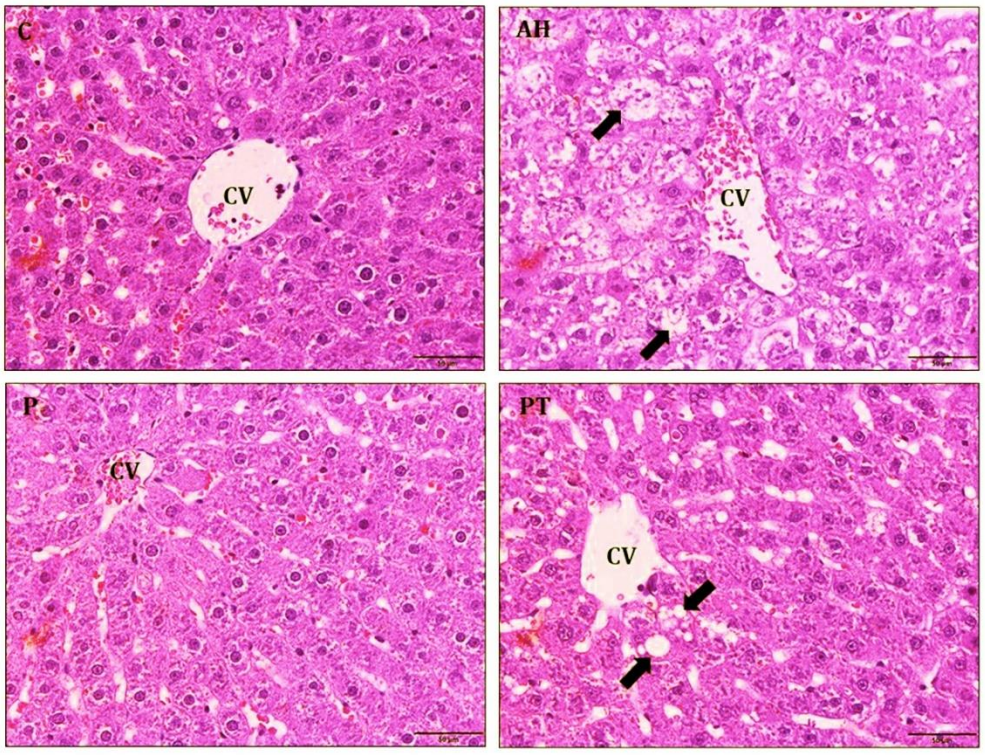

Figure 37 Histology of liver tissue was an estimate by hematoxylin and eosin staining in hepatitis rats (n=6 rats).

Normal control (C) and probiotic control (P) showed normal hepatocyte, regularity hepatocyte arrangement. Alcoholic hepatitis (AH) indicated that fat accumulation was in the hepatocyte (black arrow) and improved by probiotic *L. paracasei*

MSMC39-1 treatment (PT). 400x magnification and scale bar 50 μm. The tip of portal lobules was central vein (CV).

15. Effect of *Lactobacillus paracasei* MSMC39-1 on gut microbiota alteration in alcohol-induced hepatitis rat

Stool were collected after the end experiment for the detection of gut microbiota alteration. The data from next generation sequencing of stool revealed an increase in Bacteroidetes and a decrease in Fermicutes in alcohol hepatitis rats shown in Figure 38. After *L. paracasei* MSMC39-1 treatment in alcohol-induced hepatitis rats, the number of phylum Fermicutes (*Lactobacillus* spp.) increased whereas the number of Bacteroidetes (*Prevotella* spp.) decreased when compared to alcohol control as shown in Figure 38-39. The normal control and probiotic control showed similar number of genus *Lactobacillus* as shown in Figure 39.

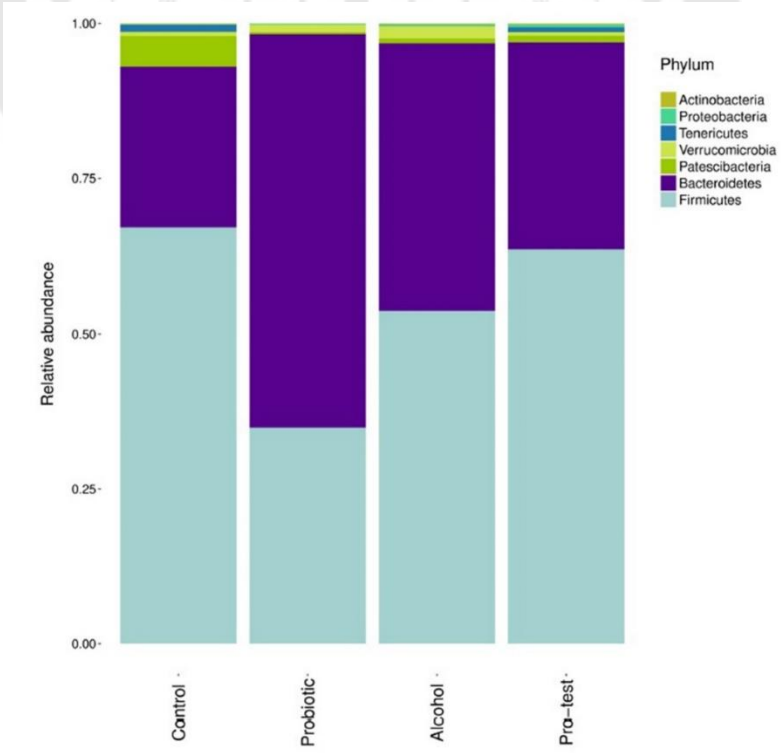

Figure 38 Effect of *L. paracasei* MSMC39-1 on gut microbiota alteration in hepatitis rats.

Statistical analysis used One way ANOVA and *ρ < 0.05; significant differences from the control (n =6 rats).

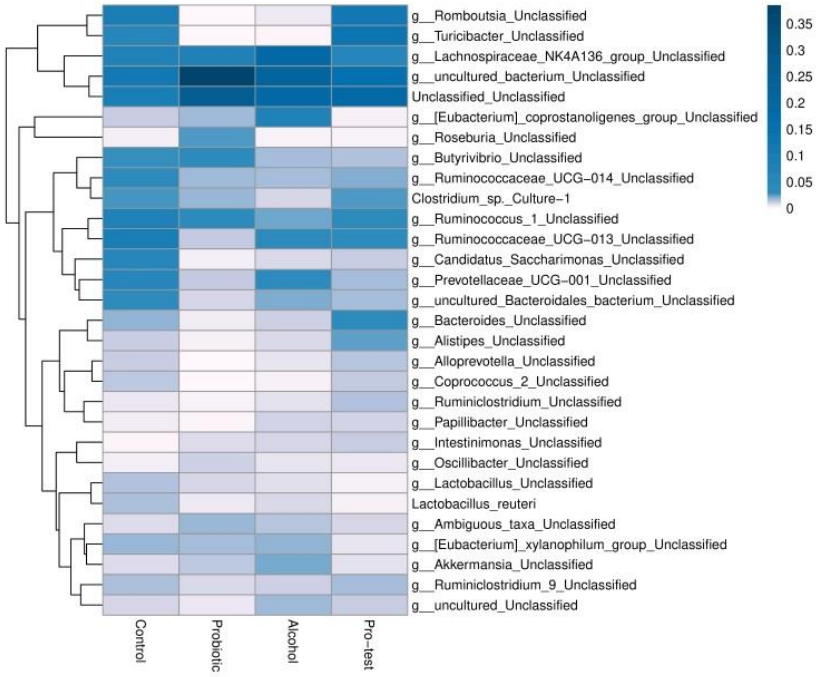

Figure 39 Heat map of *L. paracasei* MSMC39-1 on gut microbiota alteration in hepatitis rats

Statistical analysis used One way ANOVA and *ρ < 0.05; significant differences from the control (n =6 rats).

CHAPTER 5

SUMMARY DISCUSSION AND SUGGESTION

Probiotic lactic acid bacteria mostly genus *Lactobacillus* and *Bifidobacterium* have been used to promote human health. Probiotic *Lactobacillus* isolated from human, fermented foods and dairy products has good properties including anti-microbial, anti-cancer, anti-oxidant, cholesterol lowering especially immunomodulation[44, 134-136].

Alleivation of TNF – α, an initial pro-inflammatory cytokine, can be found in inflammation diseases such as inflammatory bowel disease, rheumatoid arthritis, alcoholic liver disease, atopic dermatitis, and psoriasis. TNF-α plays a key role in liver inflammation[137], alcohol liver disease and colitis [138]. Inhibition of TNF -α production by probiotic have been reported. *L. rhamnosus*, 4B15 and *L. gasseri* 4M13 isolated from human feces with anti-oxidant and cholesterol reduction properties suppressed the production of pro-inflammatory cytokine including TNF-α, IL-6, IL-1β in RAW 264.7 murine macrophage cell line[139]. In addition, surface layer protein of *L. acidophilus* decreased inflammatory cytokine secretions including TNF-α, IL-10, IL-1β, IFN-α and IFN-β in LPS activated RAW 264.7 cells[102].

In this study, probiotic lactic acid bacteria were determined in the suppression of TNF-α secretion in Caco2 cells and HepG-2 cells. Caco-2 cells, a model of enterocyte of the human intestine have been used to study epithelial barrier function [140]. HepG-2 cells is a model of hepatocyte with lipopolysaccharide (LPS) activation[141]. Lipopolysaccharide is the endotoxin, found in cell wall of gram negative bacteria such as *Escherichia* coli. Binding of LPS to toll like receptor-4 in immune cells activates pro-inflammatory cytokine production during infection and inflammation[142]. Four antigen patterns of probiotic lactic acid bacteria are probiotic culture supernatant, heat killed cells which is a model of probiotic death, sonicated cells which is probiotic cell lysis and viable cells which is a model of human eat probiotic cells. In *in vitro* study, supernatant of *L. paracasei* MSMC39-1 and *L. casei* MSMC39-3 slightly inhibited TNF-α in LPS activated HepG-2 cells and Caco-2 cells. This result is in agreement with the previous report that

supernatant of *L. plantarum* and *L. fermentum* inhibited TNF-α production in LPS induced THP-1 monocytic cells[143]. It has been reported that extracellular metabolites of *L. plantarum* prepared from supernatant suppressed TNF-α secretion via attenuation of NF-kB activation in LPS activated RAW 264.7 macrophage cell line[144]. Supernatant of *L. acidophilus*, *L. casei*, *Lactococcus lactis*, *L. reuteri*, and *Saccharomyces boulardii* decreased expression of IL-8 in HT-29 cells [145]. *Lactobacillus reuteri* supernatant reduced TNF-α and IL-6 in LPS activated RAW 264.7 macrophage cell line[146]. In this research, viable cells of MSMC39-1, MSMC39-3 and MSMC57-1, heat killed cell of MSMC39-1, MSMC39-3 and sonication cell of MSMC57-1 significantly suppressed TNF-α secretion in Caco-2 cells. Viable cells and heat killed cells of *L. paracasei* MSMC39-1 and sonicated cells of *Weissella confusa* MSMC57-1 significantly suppressed TNF-α secretion in HepG-2 cells. Similar with our results, previous study showed that heat-killed cells and sonication cells of probiotic *Lactobacillus* reduced TNF-α, and IL-6 secretion in LPS stimulated HepG-2 cells[147]. Recent study revealed that peptidoglycan extracts of *L. plantarum* isolated from human feces modulated TNF-α, and IL-6 in LPS induced murine RAW 264.7 cells[148]. This study, heat-killed cells of *L. confusus* MSMC57-1 activated TNF-α secretion in HepG-2 cells similarly to previous study showed heat killed cells of *L. brevis* induced IL-1β and IL-6 expression without LPS in RAW 264.7 cells[149]. Beneficial effects of probiotic bacteria on the immunomodulation such as heat killed cells of *L. paracasei*, *Bifidobacterium bifidum* and *Streptococcus thermophilus* were enhanced IgA production [150-152]. Moreover, spore of *B. coagulans* MTCC 5856 reduced IL-8 and induced anti-inflammatory cytokine (IL-10) secretion in LPS-induced HT-29 cells[153]. Probiotics from kefir activated IL-10, TNF-α, IL-17, IL-1β in diabetes rats[154]. Live cells of *Bifidobacterium longum* protect lung damage by activated IL-10 production in *Klebsiella pneumoniae* infection mice[155]. *L. paraplantarum* MTCC 9483 have anti-inflammatory activity by increased IL-4, IL-10 gene expression in Caco-2 cells induced by LPS and *M. luteus* ATCC 9341[32].

Thus, our results suggested that TNF-α secretion in Caco-2 cells and HepG-2 cells was suppressed by viable cell and heat killed cell of probiotic *L. paracasei*

MSMC39-1, *L. casei* MSMC39-3 and sonicated cell of *Weissella confusa* MSMC57-1. Viable cell of *L. paracasei* MSMC39-1 was used in colitis and hepatitis rat models due to its strongest suppression effect on TNF-α secretion in Caco-2 cells and HepG-2 cells. Heat killed cells and sonicated cells reduced TNF in Caco-2 cells and HepG-2 cells because cell wall components such as peptidoglycans, lipoteichoic acids, or heat labile pili in cell wall components have key role in immunomodulatory[156]. Viable cells of *L. paracasei* MSMC39-1 reduced TNF because viable cells produced metabolite and immunomodulation [155].

Dextran sulfate sodium (DSS) is a reagent used for induction of ulcerative colitis. DSS disrupts barrier function of epithelial cells leading to increase in intestinal permeability and antigen and bacteria penetration into the lumen. This activates immune cells to secrete pro-inflammatory cytokine causing intestinal inflammation[157]. Symptoms after DSS consumption include body weigh loss, bloody diarrhea, diarrhea, ulcer of epithelial cell, goblet cells reduction, intense of infammatory cells, crypt edema, colon shorten in the colon histology and microbiota alteration[158].

In the colitis experiment, disease activity indexs are body weight, stool and rectal bleeding as score of colon inflammation. The probiotic *L. paracasei* MSMC39-1 was improved disease activity indexs and the severity of colitis. An increase in TNF-α secretion in colon and liver tissue of colitis rats was significantly reduced by administration of probiotic *L. paracasei* MSMC39-1. In addition, *L. paracasei* MSMC39-1 improved colon and liver inflammation in these rats. *L. paracasei* MSMC39-1 treated colitis rats showed less ulceration and inflammation of colon and liver tissues and less fat foam in the hepatocyte. Similar results have been pevously reported by several groups. *Bifidobacterium breve* CCFM683 ameliorated colitis by reduced disease activity, increased colon length and reduced expression of TNF-α, IL-1β, IL-6 in colon tissue of DSS induced colitis mice[105]. Recent study reported that viable cells and heat killed cells of *Lactobacillus plantarum* Zhang LL ameliorated disease activity index of colitis, reduced proinflammatory cytokine in serum and colon in 5% DSS induced colitis rats[159]. *Lactobacillus casei* LC2W improved symtoms of colitis induced with *Escherichia coli*

O157:H7 by protected tight junction and reduced IL-1β, TNF-α and IL-6 in colon tissue[160]. *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in yogurt alleviated symtoms colitis in mices and induced IL-2, IL-4 production in lymph node and spleen resulting in modulated helper T cells[161]. Similar to previous studies, synbiotic LGG and prebiotic tagatose redued colitis symtoms in DSS induce colitis mice[162]. *Lactobacillus fermentum* improved colitis, reduced pro-inflammatory cytokine such as TNF- α, IL-1β, IL-6 in serum and induced anti-inflammatory cytokine (IL-10) expression in colon of DSS induced colitis in mice[163]. Furthermore, wheat germ-apple fermented with *L. delbrueckii* subsp. *bulgaricus*, *L. paracasei*, *L. plantarum* subsp. *plantarum*, and *L. helveticus*, *L. plantarum*, probiotic mixed with *Bifidobacterium infantis*, *L. acidophilus*, *Enterococcus faecalis* and *Bacillus cereus* inhibited pro-inflammatory cytokine production in colon tissue and protected tight juction in colitis model[164-166].

Microbiota modulation was observed in rats with oral administration of viable cells of *L. paracasei* MSMC39-1. Although the number of phylum Bacterioidetes was more than phylum Firmicutes in these rats, heat-map showed an increase in genus *Lactobacillus* in probiotic treated rats when compared to colitis control group. An increase in genus *Clostridium* and a decrease in genus *Lactobacillus* were observed in colitis control when compared to probiotic treated groups. Similarly, *Lactobacillus plantarum* Zhang LL, *Bifidobacterium breve* CCFM683, yogurt mixed with *L. bulgaricus* and *Streptococcus thermophilus*, synbiotic of *L. rhamnosus* GG, probiotic mixed with *B. infantis*, *L. acidophilus*, *Enterococcus faecalis* and *B. cereus* increased genus *Lactobacillus*, *Bifidobacterium* in DSS colitis model[159, 161, 162, 166, 167]. Thus, dysbiosis in GI tract which is caused from reduction of microbiota diversity including Fermicutes and Bacterioidetes can be protected by probiotics[168].

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are biomarker enzymes of liver function. Index of alcoholic hepatitis which is calculated from the ratio of AST and ALT is greater than or equal to two[169]. In this study, body and liver weight were examined in hepatitis rat model. A decrease in body weight, an increase in liver weight and an upward trend of liver-to-body weight ratio (%) were observed in rats

with alcohol administration for 8 weeks. In accordance with HUANG *et al* in 2019, probiotic increased body weight, reduced AST, ALT, IL-6, TNF-α and modulated intestinal microbiota in alcohol liver disease model[170]. Moreover, probiotics feeding increased body weight and decreased ALT, AST, IL-1β and TNF-α compared to alcohol group of normal diet and high fat diet in mice[113]. AST levels of alcohol hepatitis rats were two times more than ALT level in serum. In addition, probiotic treatment group with viable cells of *L. paracasei* MSMC39-1 showed reductions of AST and ALT levels in serum of hepatitis rat. Similar to other studies, *L. plantarum* C88, *L. plantarum*, *L. fermentum*, *L. helveticus* and *L. casei* reduced ALT and AST enzymes in the serum of liver injury model[27, 171-173]. Likewise, AST and ALT enzyme levels were decresed in alcoholic liver disease patients who received probiotics *B. bifidum* and *L. plantarum* 8PA3 supplementation[174].

In the present, TNF -α secretion (*ρ < 0.05) in liver tissue was significantly suppressed in alcohol hepatitis rats treated with probiotic *L. paracasei* MSMC39-1. This result is similar to previous studies reported that *L. plantarum*, *L. fermentum* and *L. helveticus* decreased TNF-α, IL-1β gene expression in hepatic tissue of acute liver injury models[171, 172]. Moreover, *B. longum* LC67 and *L. plantarum* LC27 reduced TNF-α level in serum and liver of ethanol induced acute liver damage in mice[175]. Wang and colleague revealed that pretreatment with *L. casei* Zhang reduced TNF-α expression in LPS /D-galactosamine induced liver injury rat[176]. Moreover, in liver fibrosis rats, *Lactobacillus* sp. and α-lipoic acid reduced TNF-α expression in liver tissue[111]. Ethanol consumption destroys gut barrier function leading to elevation of LPS in GI tract. This LPS then binds to TLR-4 on kupffer cells resulting in pro-inflammatory cytokine production and liver inflammation[177]. There is a study showed that probiotics reduce pro-inflammatory cytokine such as exopolysaccharides produced by *L. buchneri* TCP016, reduce pro-inflammatory cytokine such as TNF-α, IL-1β, IL-6, protect liver injury in lipopolysaccharide or D-galactosamine-induced liver damage in mice[178]. Supernatant of *L. reuteri* reduced liver inflammation by inhibition of IL-6 and TNF-α production in acute liver injury mice[179].

Chronic alcohol consumption induces lipid accumulation via AMP-activated protein kinase (AMPK) signaling pathway, resulting in hepatic steatosis[180]. Hepatic steatosis and inflammatory cells infiltration can be observed in liver histology of alcohol hepatitis rats. On the other hand, feeding viable cells of *L. paracasei* MSMC39-1 reduced hepatic steatosis and number of inflammatory cells. Mechanism of action of probiotic in reduced hepatic steatosis may be due to reduction of sterol regulatory element-binding protein 1 (SREBP-1), which plays a key role in lipogenesis and fatty acid oxidation[180].

Previous animal model showed that oral gavage with *L. rhamnosus* GG improved leaky gut, reduced colonic and liver inflammation in alcohol-induced liver injury in rats[131]. Similarly, Wang Y and coworkers indicated that cell free supernatant of *L. rhamnosus* GG improved liver damage and intestinal inflammation by prevent tight junction[181]. In contrast, administration of probiotics *L. casei*, *L. plantarum*, *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus*, *B. longum*, *B. breve*, *B. infantis* and *Streptococcus salivarius* subsp. *thermophilus* (VSL#3) for 6 months reduced hepatic encephalopathy in liver cirrhosis Indian patients[182].

Furthermore, in this study *L. paracasei* MSMC39-1 was a potent gut microbiota modulator which increased genus *Lactobacillus* in alcohol induced hepatitis rats. There was a study showed that *L. buchneri* TCP016 modulated gut microbiota in lipopolysaccharide/D-galactosamine-induced liver damage in mice[178]. Recent study reported that pretreated live cells with probiotic *B. breve* for 6 weeks was able to modulate gut microbiota in alcohol liver disease mice model via protection of intestinal tight junction and liver inflammation[183]. Likewise, supplementation of *L. rhamnosus* (LGG) modulated intestinal microbiota and alleviated liver injury[184].

Suppression of TNF-α secretion in probiotic is strains specific or strain-dependent. Thus, further studies should be done in order to understand the correlation and role of probiotic lactic acid bacteria in inhibition of TNF-α secretion. Probiotic strains with potent anti-inflammation in colitis and hepatitis can be used as prevention and treatment of various disorders caused by inflammation. Therefore, our results suggest commercial benefits of these probiotic strains. It is possible that these strains can be used

in Thai food and beverage market in order to reduce probiotic stratins imported from other countries.

Conclusion

In conclusion, the finding suggested that probiotic *Lactobacillus paracasei* MSMC39-1 inhibited TNF-α production in Caco-2 cells and HepG-2 cells, improved colon and liver inflammation. Administration of *L. paracasei* MSMC39-1 modulated microbiota by increased number of *Lactobacillus* in DSS-induced colitis rats and alcohol-induced hepatitis rats.

Further studies should be conducted to confirm the protective roles and other functions of *L. paracasei* MSMC39-1 in alcoholic liver disease and colitis induced inflammation condition.

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