Identification of probiotic bacteria with anticancer activity against colorectal cancer *in vitro*

Miss Patcharin Prakobwat

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การค้นหาเชื้อแบคทีเรียโพรไบโอติกส์ที่มีคุณสมบัติในการยับยั้งเซลล์มะเร็งลำไส้ใหญ่และทวารหนักในหลอดทดลอง

น.ส.พัชรินทร์ ประกอบวัฒน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	Identification of probiotic bacteria with anticancer activity against colorectal cancer *in vitro*
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พัชรินทร์ ประกอบวัฒน์ : การค้นหาเชื้อแบคทีเรียโพรไบโอติกส์ที่มีคุณสมบัติในการยับยั้งเซลล์มะเร็งลำไส้ใหญ่และทวารหนักในหลอดทดลอง. (Identification of probiotic bacteria with anticancer activity against colorectal cancer *in vitro*) อ.ที่ปรึกษาหลัก : รศ. ดร.พญ.กนิษฐา ภัทรกุล, อ.ที่ปรึกษาร่วม : รศ. ดร.สมหญิง ธัมวาสร

มะเร็งลำไส้ใหญ่และทวารหนัก (colorectal cancer) เป็นสาเหตุของการเสียชีวิตจากโรคมะเร็งเป็นอันดับสามของโลกและในประเทศไทย ปัจจัยเสี่ยงหนึ่งของการเกิดมะเร็งลำไส้ใหญ่คือการเสียความสมดุลของเชื้อจุลชีพภายในลำไส้ใหญ่ เชื้อจุลชีพที่มีประโยชน์ต่อร่างกาย เช่น เชื้อแลคโตบาซิลลัส มีคุณสมบัติในการปรับสัดส่วนเชื้อจุลชีพในลำไส้ใหญ่ให้กลับมาสมดุล การศึกษานี้มีวัตถุประสงค์เพื่อค้นหาเชื้อแลคโตบาซิลลัสสายพันธุ์ที่มีคุณสมบัติในการยับยั้งเซลล์มะเร็งลำไส้ใหญ่และทวารหนักในหลอดทดลอง โดยคัดเลือกจาก 39 สายพันธุ์ที่มีการศึกษาก่อนหน้านี้ว่าน้ำเลี้ยงเชื้อมีฤทธิ์ในการยับยั้งการอักเสบ การทดลองนี้นำน้ำเลี้ยงเชื้อแลคโตบาซิลลัสทั้งหมด 39 สายพันธุ์ที่ระดับความเข้มข้นต่างกันและ/หรือ ปรับความเป็นกรด-ด่าง มาบ่มร่วมกับเซลล์มะเร็งลำไส้ใหญ่ทั้งสองชนิด คือ HT-29 และ Caco-2 เป็นเวลา 24, 48 และ 72 ชั่วโมง จากนั้นหาจำนวนเซลล์ที่รอดชีวิตด้วยวิธี 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide (MTT assay) ผลการทดลองพบว่า น้ำเลี้ยงเชื้อจาก 2 สายพันธุ์ คือ *L. vaginalis* L19 และ *L. gasseri* L20 สามารถลดอัตรารอดชีวิตของเซลล์อย่างมีนัยยะสำคัญทางสถิติที่บางระดับความเข้มข้นและเวลาในการบ่ม เนื่องจากการยับยั้งการเจริญของเซลล์มะเร็งด้วยน้ำเลี้ยงเชื้อแลคโตบาซิลลัสอยู่ในระดับต่ำ จึงทำการศึกษาต่อโดยนำเชื้อแลคโตบาซิลลัสที่ยังมีชีวิตมาบ่มกับเซลล์มะเร็งชนิด Caco-2 โดยใช้สัดส่วนของจำนวนเชื้อต่อเซลล์ (MOI) ที่แตกต่างกัน และประเมินการรอดชีวิตของเซลล์ด้วยวิธี flow cytometry พบว่าเชื้อสายพันธุ์ *L. gasseri* L20 สามารถลดการรอดชีวิตของเซลล์ได้สูงและมากกว่าสายพันธุ์ *L. vaginalis* L19 อย่างมีนัยยะสำคัญทางสถิติเมื่อใช้สัดส่วนเชื้อต่อเซลล์ที่ MOI 100 ภายหลังการบ่มนาน 24 และ 48 ชั่วโมง ในการทดสอบความสามารถในการยึดเกาะต่อเซลล์ชนิด Caco-2 ที่มีการพัฒนาจนเปลี่ยนแปลงผิวเซลล์จนคล้ายผนังเซลล์ลำไส้แล้ว โดยอาศัยวิธีการทดสอบการยึดเกาะและการดูด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด พบว่าเชื้อทั้งสองสายพันธุ์สามารถเกาะผิวเซลล์ได้แม้จะอยู่ในระดับต่ำ ดังนั้น เชื้อ *L. vaginalis* L19 และ *L. gasseri* L20 สองสายพันธุ์นี้อาจจะเป็นประโยชน์ในการนำไปใช้เพื่อป้องกันมะเร็งลำไส้ใหญ่ต่อไปในอนาคต

สาขาวิชา	จุลชีววิทยาทางการแพทย์	ลายมือชื่อนิสิต .....
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KEYWORD: Colorectal cancer, Probiotic, Anticancer activity, Anti-proliferation effect, Bacterial adhesion

Patcharin Prakobwat : Identification of probiotic bacteria with anticancer activity against colorectal cancer *in vitro*. Advisor: Assoc. Prof. Dr. KANITHA PATARAKUL, M.D. Co-advisor: Assoc. Prof. Dr. SOMYING TUMWASORN

Colorectal cancer is the third most common cause of cancer death worldwide. It is ranked third for new cancer patients in Thailand. Colorectal cancer is associated with multifactorial risk factors including the imbalance of gut microbiota. This project aimed to identify *Lactobacillus* strains isolated from Thai healthy populations that have anticancer activities against colorectal cancer *in vitro*. *Lactobacillus* cultured media (LCM) obtained from thirty-nine *Lactobacillus* strains, previously shown to have anti-inflammatory activity, were selected and used at different concentrations and/or pH adjustment to determine the anti-proliferative effect on HT-29 and Caco-2 colon cancer cells at 24, 48, and 72 h. The cell viability was analyzed by 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide (MTT assay). LCM from 2 out of 39 strains, *L. vaginalis* L19 and *L. gasseri* L20, significantly reduced cell viability at certain concentrations and time-points. Due to weak anti-proliferative activity of LCM, viable *L. vaginalis* L19 and *L. gasseri* L20 were used to treat Caco-2 cells and cell viability was determined by flow cytometry. *L. gasseri* L20 at the MOI of 100 strongly and significantly reduced Caco-2 cell viability more than *L. vaginalis* L19 after 24 and 48 h incubation. Furthermore, *L. vaginalis* L19 and *L. gasseri* L20 could weakly adhere to polarized and differentiated Caco-2 cells, i.e., enterocyte-like epithelial cells, as demonstrated by the adhesion assay and scanning electron microscopy. Therefore, *L. vaginalis* L19 and *L. gasseri* L20 might be useful for prevention of colorectal cancer in the future.

Field of Study:	Medical Microbiology	Student's Signature .....
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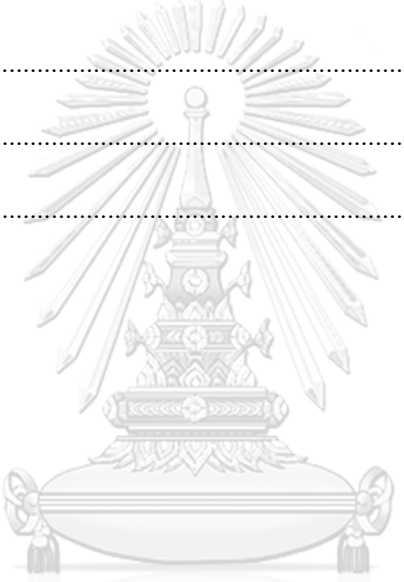

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ABBREVIATION

CRC	Colorectal Cancer
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
LCM	Lactobacillus Conditioned Medium
MOI	Multiplicity of infection
MRS	De Man, Rogosa and Sharpe agar
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
MTT	2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide
OD	Optical Density
PBS	Phosphate-Buffered Saline
SEM	Scanning Electron Microscope
7-AAD	7-amino-actinomycin D

CHAPTER I

INTRODUCTION

Colorectal cancer (CRC) is an important public health issue. According to the World Health Organization (WHO) in 2018, CRC was the third most common cancer and the second leading cause of death among different types of cancer death worldwide. CRC is also a common cancer in Thai patients. From the hospital-based cancer registry of Thailand in 2018, it was ranked the first and the third most common cancer in males and females, respectively. CRC patients tend to increase continuously and are frequently found in populations aged over 50 years old. CRC occurs in a multi-step progression starting with abnormal cell proliferation leading to the development of adenomatous polyps and progressing to CRC at the end (1). CRC development is associated with multifactorial risk factors such as genetic alteration, family history, advanced age, a personal history of gastrointestinal disease (including inflammatory bowel disease, polyps), lifestyle, dietary pattern, and gut microbiota profile (1, 2). Recently, numerous research studies have revealed that gut microbiota are associated with intestinal tumorigenesis (3). Gut microbiota are composed of approximately 100 trillion microorganisms (including mostly bacteria, fungi, and viruses). In normal circumstances, the gut microbiota has a balanced composition between beneficial and pathogenic bacteria in a homeostasis modulation. A meta-analysis study demonstrated that the bacterial phyla composition of gut microbiota

were different between CRC patients and healthy (4). In contrast to beneficial bacteria, pathogenic bacterial populations tend to increase in CRC patients. Therefore, the imbalance of gut microbiota (dysbiosis) may be associated with the development of CRC. To prevent CRC development, improving beneficial bacteria, preventing gut dysbiosis, and recovering gut homeostasis has received growing attention.

Probiotics are live beneficial microorganisms that give health benefits to the host when received in an adequate amount. Several investigations have demonstrated that probiotics have efficacy to modulate gut flora and host immune system in the gastrointestinal tract (5). Recent studies found that probiotics could be used to prevent and treat many gastrointestinal disorders or diseases, such as *Helicobacter pylori* or *Clostridium difficile* infection, and irritable bowel syndrome (6). Lately, the ability of probiotics in CRC prevention has been demonstrated by many mechanisms, for example, competition with pathogenic gut flora, reduction of DNA damage, improvement of the intestinal barrier, immunomodulation, anti-inflammation, and anti-proliferation of cancer cells (7). However, probiotics from the same genus and species may have different effects on the host due to strain-specific properties. Only certain strains can persist in individual's gut. Thus, each probiotic strain should have potential effects on the host in a case-by-case manner (8).

In our previous studies, new lactobacillus strains isolated from infant feces and gastric biopsies were shown to have anti-inflammatory activity including suppression of IL- 8 production induced by *Clostridium difficile* infection in HT-29 cells (9), inhibition of TNF production in LPS-activated THP-1 monocytoid cells, and inhibition of IL-8 production in *H. pylori*-stimulated AGS gastric epithelial cells (10). However, the property of these *Lactobacillus* strains on anticancer activity other than anti-inflammatory activity has never been characterized. This study aimed to identify new *Lactobacillus* strains with anticancer activity against CRC *in vitro* such as anti-proliferative activity of *Lactobacillus* conditioned medium (LCM) and viable lactobacilli against CRC cell lines, and ability to adhere to epithelial cells. This study used two CRC cell lines, HT-29 cells, and Caco-2 cells, which have been widely used to study the function of human intestinal cells *in vitro.* These *Lactobacillus* strains that have anticancer activity may be useful for prevention or co-treatment of CRC patients in the future.

CHAPTER II

OBJECTIVE

Hypothesis

Probiotic strains isolated from Thai healthy populations have anticancer activities against colorectal cancer including anti-inflammation, anti-proliferation activity of colon cancer cells, and/or adhesion to colon epithelial cells.

Objective

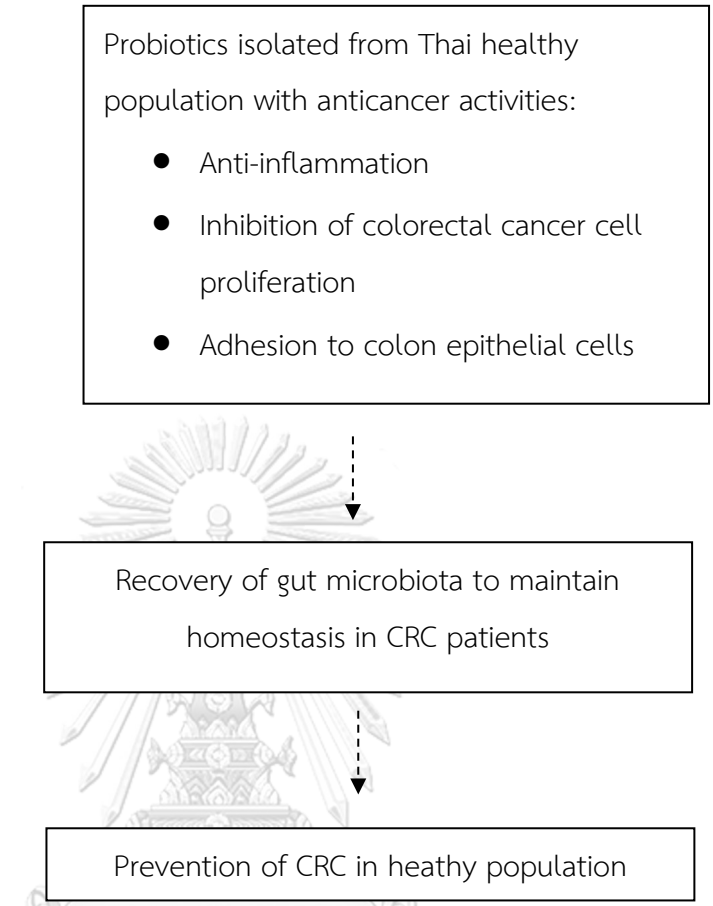
To identify probiotic strains isolated from Thai healthy populations that have anticancer activities against colorectal cancer *in vitro* including

Anti-inflammation

Anti-proliferation of colon cancer cells

Adhesion to colon epithelial cells

Conceptual Framework



--------indicate future research and application

CHAPTER III

REVIEW OF RELATED LITERATURE

Colorectal cancer

Colorectal cancer (CRC) is a lethal cancer that occurs in the lower portion of the digestive system involving the large intestine or rectum. According to GLOBOCAN 2018, CRC is the third most commonly diagnosed cancer among any types of cancer and the second most deadly cancer worldwide causing 881,000 deaths (Figure 1) (11). The incidence rate of CRC has been gradually rising worldwide, especially in developing countries. The prevalence of CRC is high in western countries and also increases in developed Asian countries (12). CRC is also considered as an important public health problem in Thailand. It is one of the top 3 most common cancers among Thai patients. According to the hospital-based cancer registry of Thailand in 2018, it was ranked the first and the third most common cancer in males and females, respectively. In addition, CRC frequently occurs in the population aged over 50 years old.

Mostly, CRC develops progressively from adenomatous polyp or adenoma to invasive cancer by a multi-step process. The process is driven by many risk factors such as i) heredity and medical history of chronic inflammatory bowel diseases including personal or family history of CRC or adenomatous polyps ii) sex iii) advanced age iv) race and v) behavior (Figure 2) (2, 13). Interestingly, well known

behavior associated with the risk factors of CRC includes lifestyle or physical activity, smoking, alcohol consumption, and western diet.

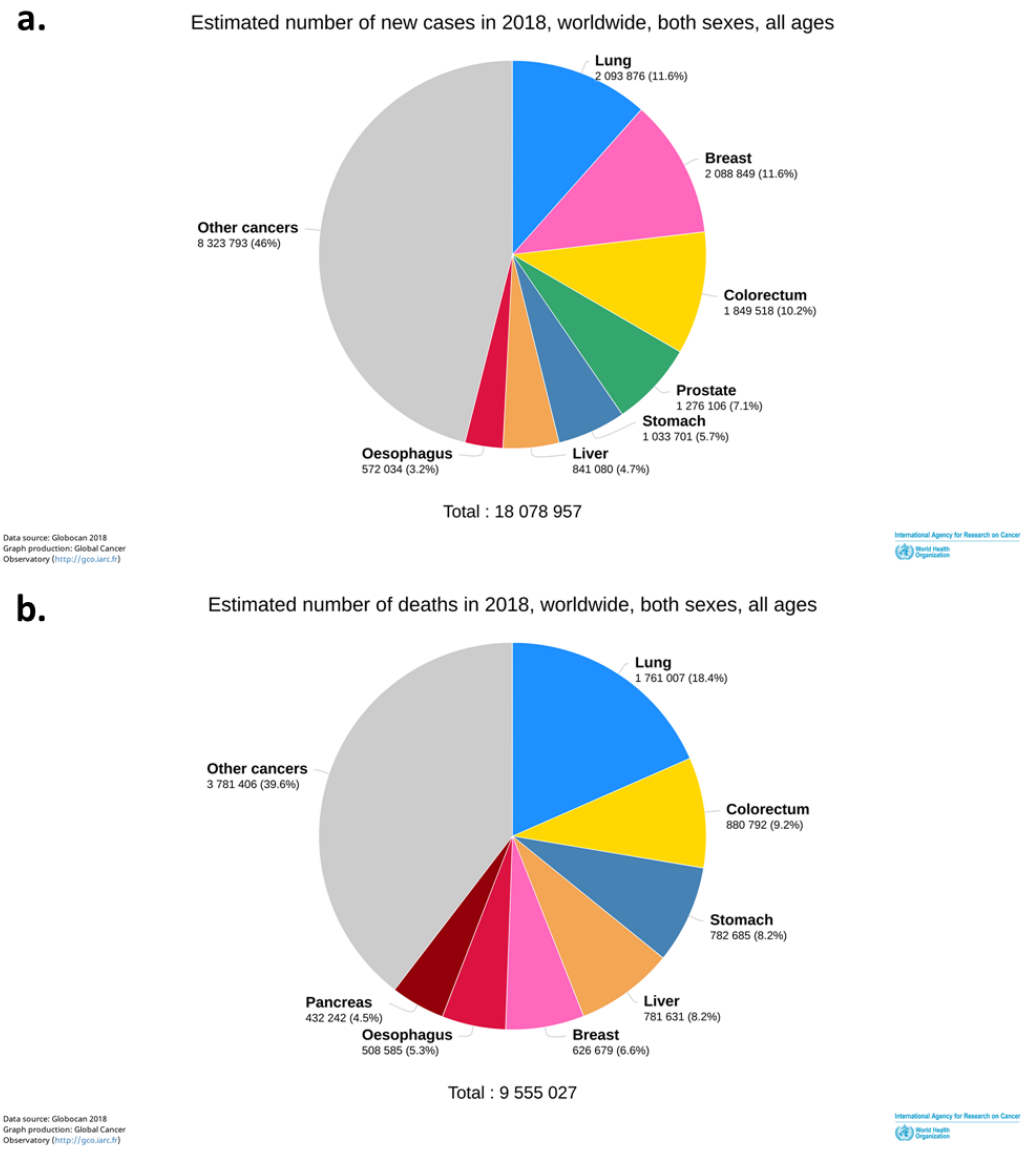


Figure 1 Incidence and mortality rates of cancers worldwide in 2018.
Data reported in 2018 by WHO demonstrate the incidence rate (a) and mortality rate (b) of cancers (14)

Recently, many researchers have focused on gut microbiota and found the relationship between diet and gut microorganisms (15). Composition of gut microbiota can be influenced by dietary change. Thus, diet may be an important modulator of gut microbiota. In addition, gut microbiota are associated with CRC progression (16). Consumption of red meat and low fibers increases risk of CRC. However, it is unclear whether the change of gut microbiota is the cause or the effect of CRC progression.

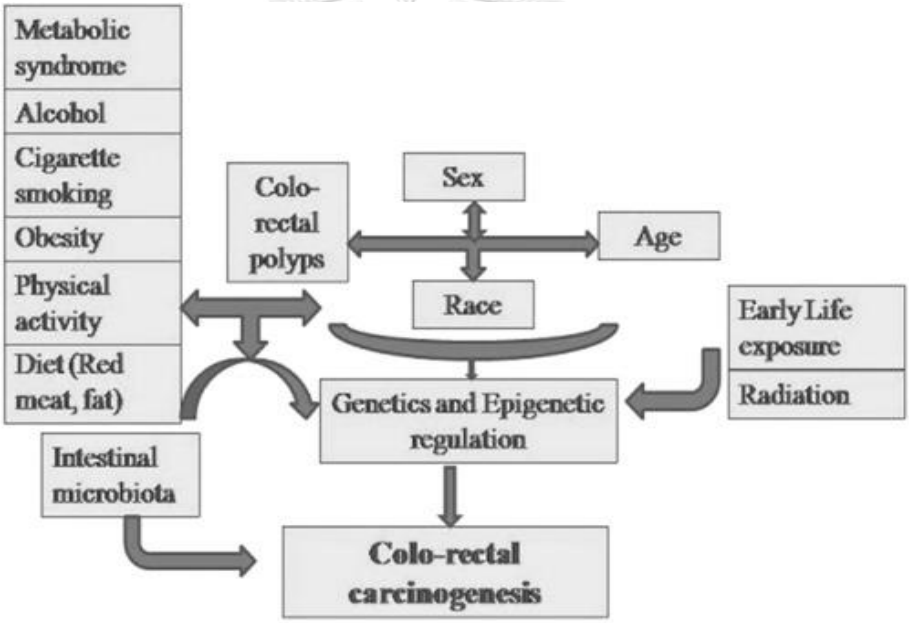


Figure 2 The risk factors associated with colorectal carcinogenesis (2).

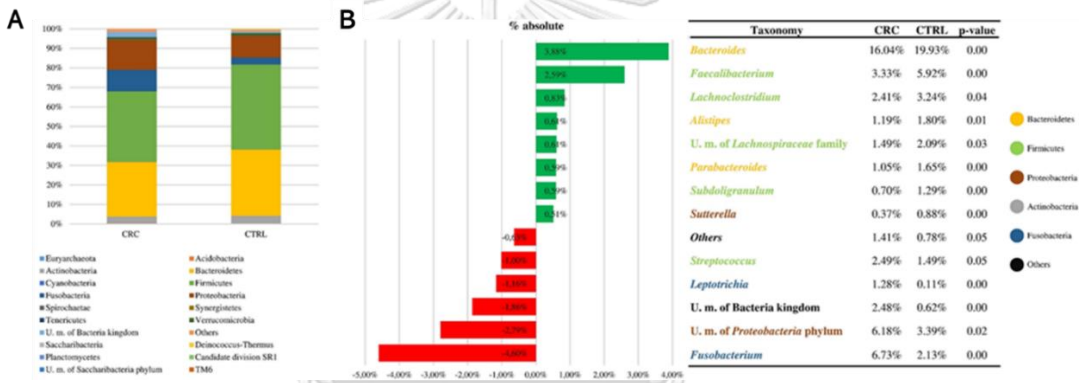
Gut microbiota

Gut microbiota is a complex system of microbial communities composed of approximately 100 trillion microorganisms including bacteria, fungi, and viruses. A previous metagenomic study showed that gut microbiota is a diverse ecosystem containing approximately 1,000–1,500 species of microorganisms (17). However, bacteria are the most abundant microorganism colonized in the gut. These commensal bacteria play key roles in maintenance of host intestinal barrier, modulation of host metabolisms including absorption of indigestible carbohydrates, producing the potential vitamins (vitamin B and K), and modulation of the host immune responses against pathogens (18). In addition, gut microbiota also supports the biotransformation of numerous chemical compounds. Gut microbiota transforms the complex nutrients components, such as undigested food fiber, non-digestible carbohydrates (NDCs) or resistant starch (RS), and mucins to sugars that are fermented to short-chain fatty acids (SCFAs) (19). SCFAs are volatile saturated fatty acids including formic, acetic, propionic, butyric, valeric, and caproic acids. The major acid found in the colon was acetate, propionate, and butyrate that play a very important role in maintaining intestinal and immune homeostasis by regulating pH, increasing the absorption of calcium, iron, and magnesium. For example, acetate and propionate enhance calcium absorption (20). SCFAs reduce pH to prevent the overgrowth of pathogenic bacteria in the gut, for example, *E. coli* or *Salmonella* spp. can be killed by propionic acid or formic acid (21). In addition, recent studies found

that butyrate induces histone hyperacetylation in numerous cells and inhibits histone deacetylase (HDAC) involved in cell cycle *in vitro* (22). Butyrate has ability to induce cell cycle arrest, differentiation and apoptosis of CRC cells (22, 23). Moreover, these acids affect the maintenance of the normal structure, integrity, and function of the intestine.

Normally, the gut microbiota has a balanced composition, called gut symbiosis or eubiosis. Gut dysbiosis, which is the alteration of microorganism community in the gut leading to the imbalance of gut microbiota, may be associated with chronic inflammatory conditions and production of carcinogenic metabolites leading to CRC (24). The meta-analysis studies comparing the microbial communities between healthy people and CRC patients demonstrated gut dysbiosis in CRC patients (4). Recent discoveries found that the gut microbiota of CRC patients contained diverse oncogenic bacteria associated with CRC development such as *Fusobacterium nucleatum, Helicobacter pylori, Enterococcus faecalis,* genotoxic *Bacteroides fragilis,* and genotoxic *Escherichia coli* (25, 26). In normal circumstances, the human gut microbiota is dominated by 4 main phyla: Firmicutes, Bacteroides, Actinobacteria, and Proteobacteria. When dysbiosis occurs, the pathogenic bacteria such as Proteobacteria and Fusobacteria increase (Figure 3). The alteration of gut microbiota may allow pathogenic bacteria to induce gene mutation and chronic inflammation that damage the natural barrier of host defenses. Pathogenic bacteria can secrete harmful bacterial enzymes that may be involved in the carcinogenic

process, such as β-glucuronidase, nitroreductase, azoreductase, 7-α-dehydroxylase, and cholesterol dehydrogenase. Thus, gut dysbiosis may be related to the multistep process from precancerous adenoma to CRC as shown in Figure 4 (2, 18, 27). Moreover, dysbiosis and reduction of the number of bacteria producing metabolites such as SCFAs, also occur in patients with other diseases such as inflammatory bowel diseases (IBD), irritable bowel syndrome (IBS), type 2 diabetes (T2D), obesity, autoimmune disorders .



Taxonomy	CRC	CTRL	p-value
Bacteroides	16.04%	19.93%	0.00
Faecalibacterium	3.33%	5.92%	0.00
Lachnoclostridium	2.41%	3.24%	0.04
Alistipes	1.19%	1.80%	0.01
U. m. of *Lachnospiraceae* family	1.49%	2.09%	0.03
Parabacteroides	1.05%	1.65%	0.00
Subdoligranulum	0.70%	1.29%	0.00
Sutterella	0.37%	0.88%	0.00
Others	1.41%	0.78%	0.05
Streptococcus	2.49%	1.49%	0.05
Leptotrichia	1.28%	0.11%	0.00
U. m. of Bacteria kingdom	2.48%	0.62%	0.00
U. m. of *Proteobacteria* phylum	6.18%	3.39%	0.02
Fusobacterium	6.73%	2.13%	0.00

Figure 3 The bacterial composition of gut microbiota in colorectal cancer patients (CRC) and healthy control (CTRL) at the phylum and the genus levels [Modified from (4)].

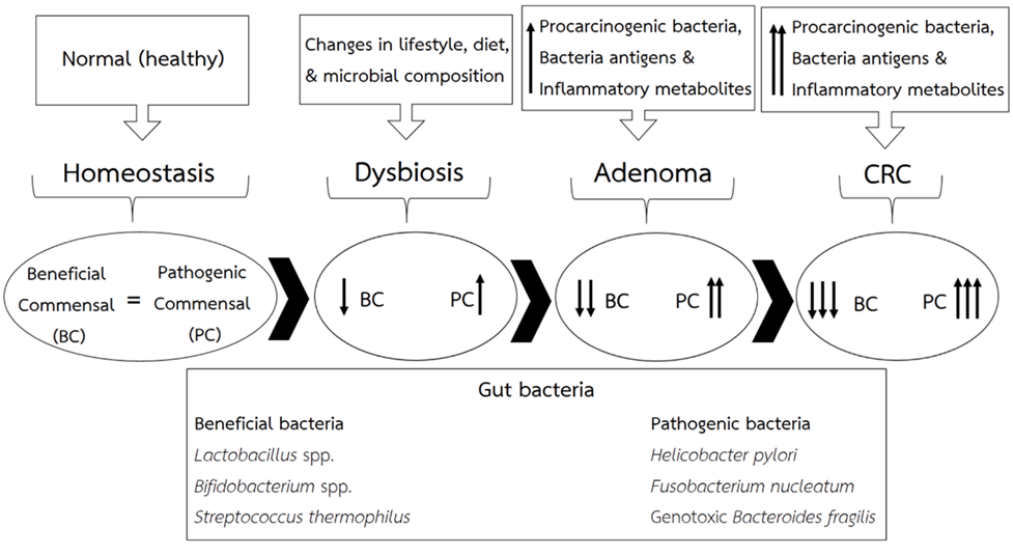


Figure 4 The multistep process of colorectal cancer development [Modified from (18)].

Probiotics

The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) define the term of probiotics as live microorganisms which confers health benefits when the administration is in adequate amounts (28). Probiotics were originally used when people regarded the beneficial health effects of the consumption of fermented foods such as bread, milk, cheese, vegetables, beer, and wine (29). The connection between fermented foods, bacteria, and health arose with the foundation of the discipline of microbiology by van Leeuwenhoeck who observed yeast cells in fermenting beer under the microscope. Thus, probiotics have been emerged to study their roles for health benefit. In the earlier part of last century, the use of fermented milk with probiotics was arised to treat patient with gastrointestinal infections. Nowadays, the focus has moved to the use of these probiotics for improvement of the gastrointestinal tract health.

The selection of effective probiotics was based on general properties of probiotics such as acid and bile salt tolerance, antimicrobial activity against pathogenic bacteria including *Salmonella*, *Shigella* and *Helicobacter*, and adhesion to mucosal and epithelial surfaces (30). Ability to adhesion to the mucosal and epithelial cell is considered as the important property leading to other potential effects including immune modulation, competitive exclusion of pathogen (31). Most probiotics belong to the natural gut microbiota and are mainly lactic acid bacteria (LAB). LAB are Gram positive bacteria, non-spore forming cocci, coccobacilli, or rod

shape (32). It has the ablity to convert fermentable carbohydrates into lactic acid as an end product. The members of LAB are a large number of bacterial genera including lactobacilli, lactococci, enterococci, streptococci, leuconostoc, and pediococci (33). These genera are classifed as LAB by morphology, pH and salt tolerance, temperature optimum, habitats, and pathogenic potential. The well-known genera among all LAB mostly studied for the potential effects and widely used in food industry are genera of lactobacilli and bifidobacteria. In general, Lactobacilli inhabit and colonize at the human vagina and gastrointestinal tract together with Bifidobacterium, the first and most commonly found bacteria to colonize the infant gut (34).

The bacterial species commonly considered as probiotics are shown in Table1. Moreover, several probiotic bacteria have been used in many commercial products including yoghurt, fermented dairy deserts, spray-dried milk powder, cheeses, ice cream, freeze-dried yoghurt, and fruit juices (35). Commercial probiotics for consumers are shown in Table 2.

Common bacteria considered as probiotics			
Lactobacillus species	*L. acidophilus*	*L. brevis*	*L. casei*
	L. crispatus	*L. delbrueckii* subsp. *bulgaricus*	
	L. gasseri	*L. johnsonii*	*L. paracasei*
	L. plantarum	*L. reuteri*	*L. rhamnosus*
Bifidobacterium species	*B. adolescentis*	*B. animalis* subsp. *lactis*	*B. bifidum*
	B. breve	*B. infantis*	*B. lactis*
	B. longum		
Other Lactic acid bacteria	*Enterococcus faecium*	*Lactococcus lactis* subsp. *lactis*	
	E. durans	*Leuconostoc mesenteroides*	
	Pediococcus acidilactici	*Streptococcus thermophilus*	
Nonlactic acid bacteria	*Escherichia coli* strain nissle	*Propionibacterium freudenreichii*	
	Saccharomyces cerevisiae	*S. boulardii*	

Table 1 The bacterial species commonly considered as probiotics [Modified from (31, 36)]

Strains		
L. acidophilus LA-1	*L. acidophilus* DDS-1	*L. salivarius* UCC118
L. paracasei CRL 431	*L. casei* DN014001 (Immunitas)	*B. longum* BB536
B. lactis Bb-12	*L. rhamnosus* GR-1	*L. acidophilus* LB
L. casei Shirota	*L. johnsonii* La1 (same as Lj1)	*L. paracasei* F19
B. breve strain Yakult	*L. plantarum* 299V	*L. crispatus* CTV05
L. acidophilus SBT-2062	*L. rhamnosus* 271	*L. casei* DN 114
B. longum SBT-2928	*L. rhamnosus* GG	*S. boulardii*
L. acidophilus R0011	*L. acidophilus* NCFM	*B. lactis* HN019 (DR10)
L. rhamnosus R0052	*L. rhamnosus* LB21	*Lactococcus lactis* L1A
L. delbrueckii subsp. bulgaricus 2038		*L. reuteri* SD2112 (same as MM2)

Table 2 Probiotic strains used as commercial products [modified from (35)].

Clinical applications of probiotics

Probiotics are beneficial microorganisms that may restore healthy microbiota and modulate homeostasis in the gut (37), stimulate and modulate the immune system, synthesize and enhance the bioavailability of nutrients (35). In the past decade, strong evidences support the efficacy of probiotics to improve the treatment of gastrointestinal infection and diseases including acute infectious diarrhea, antibiotic-associated diarrhea, irritable bowel syndrome, ulcerative colitis, necrotizing enterocolitis and Crohn's disease (Figure 5) (38).

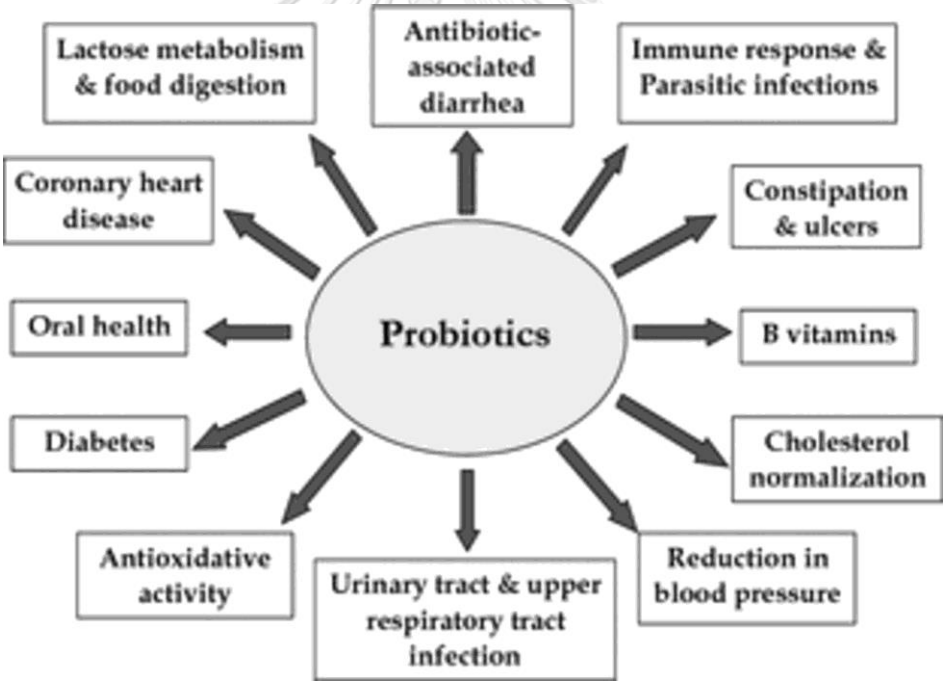


Figure 5 The potential effects of probiotics (38).

Interestingly, recent studies found that probiotics had the potential effect to prevent CRC (39). The potential mechanisms of probiotics to prevent CRC have been proposed as shown in Figure 6. During the CRC development, probiotics may have

potential effects such as prevention of oxidative stress, reduction of DNA damage, binding to mutagen, inhibition of cancer cell proliferation, and induction of apoptosis (7). Recent studies showed that probiotics can play a role in the regulation of cell apoptosis via intrinsic and extrinsic pathways as shown in Figure 7 (40). The extrinsic pathway engages Fas, tumor necrosis factor receptor, to induce caspase related pathway. For example, *L. reuteri* ATCC PTA 6475 was shown to suppress tumor necrosis factor (TNF)-dependent nuclear factor- kB (NF-kB) activation (41). In addition, *L. casei* induced apoptosis death by upregulation of TNF-related apoptosis-inducing ligand (TRAIL) (42). Probiotics also suppress cell growth and induced apoptosis cell death by intrinsic pathway that requires mitochondrial localization and activation of Bax and Bak. Cell-bound exopolysaccharides (cb-EPS) isolated from *L. acidophilus* 606 induced apoptosis by Bcl-2 and Bak, which are directly or indirectly associated with autophagic cell death (43). Moreover, several in vitro and in vivo studies supported the benefit of probiotics to reduce risk factors of CRC as shown in Table 3. Thus, probiotics supplement is currently under clinical studies as a novel approach for prevention of CRC. For example, probiotics consumed in CRC patients undergoing colon resection could decrease postoperative septic complications after surgery (44) and decreased recurrence of CRC with moderate/severe atypia (45).

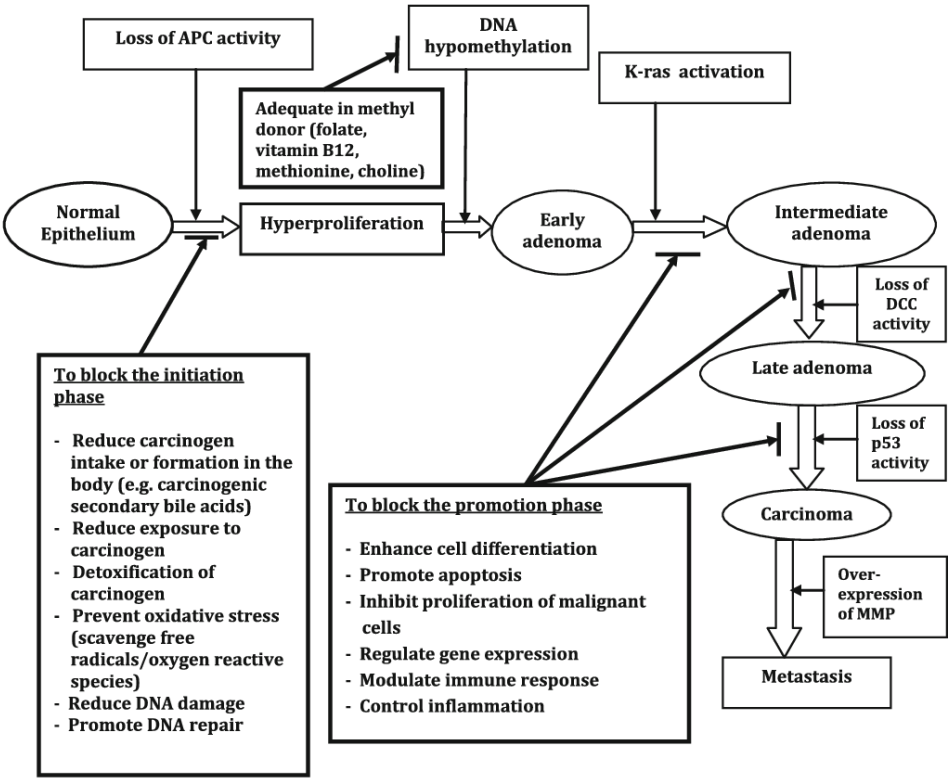


Figure 6 The possible mechanisms of probiotics to prevent the development of colorectal cancer (7).

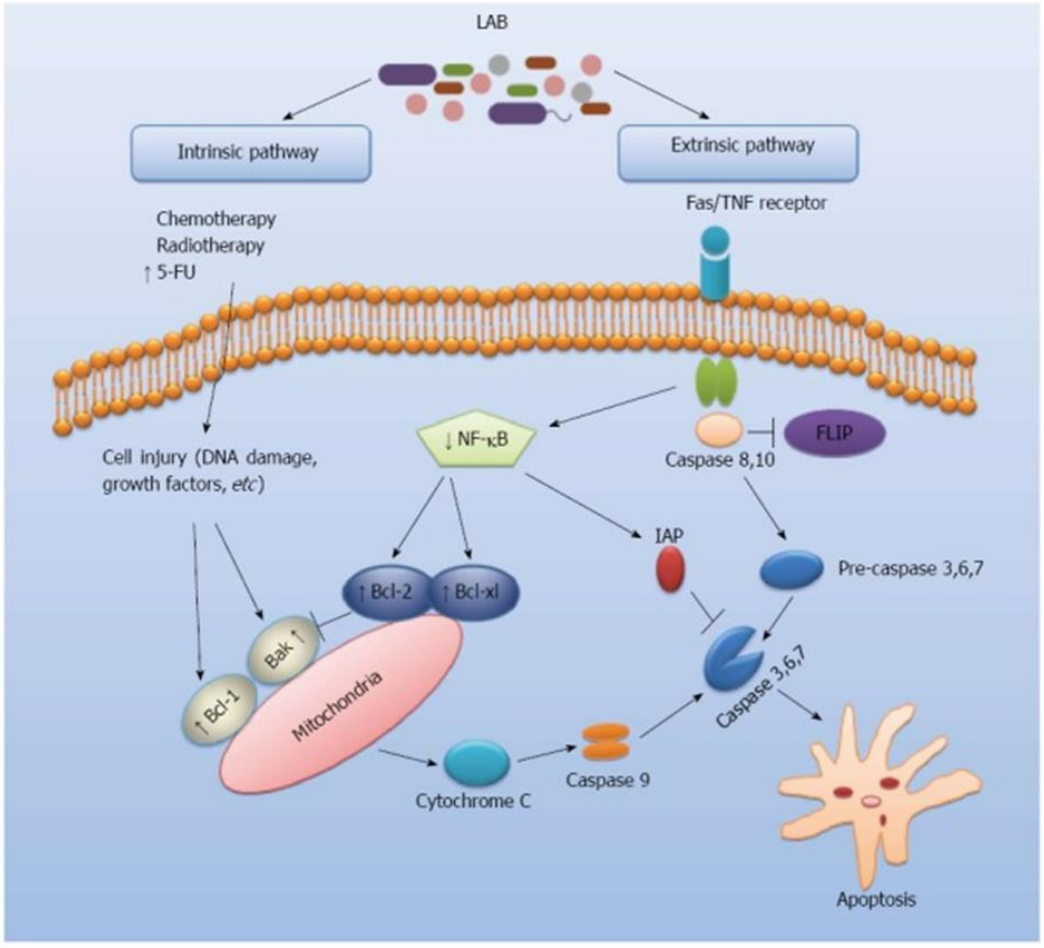


Figure 7 Potential effects of lactic acid bacteria on colorectal cancer development via extrinsic and intrinsic pathways of apoptosis (40).

Table 3 The effect and mechanisms of probiotics on colorectal cancers shown by in vitro and in vivo studies.

No.	Probiotic Bacteria	Cell lines/ animal models	Effects	Ref.
Research on cell lines/*in vitro*				
1.	*L. casei* ATCC393	CT26 cells (murine colon carcinoma), HT29 cells (human colon carcinoma)	Anti-proliferation activity. Induction of apoptosis of CT26 and HT-29 cells.	TIPTIRI-KOURPETI et al. 2016
2.	*Enterococcus faecium* RM11, *L.fermentum* RM2	Caco-2 cells	Anti-proliferation activity and strong adhesion to Caco-2 cells	Thirabunyanon et al 2009
3.	40 different probiotic bacteria isolates	Caco–2, HRT–18, Vero cell	Lactobacillus acidophilus LA102 and Lactobacillus casei LC232 showed clear cytotoxic activity with no cytotoxic activity on normal Vero cells.	AWAISHEH et al 2016
4.	*L. rhamnosus* GG	Caco–2 cells	Decreased level of IL–8	LOPEZ et al 2008
5.	*L.paracasei subsp. paracasei M5, L.paracasei subsp. paracasei X12, L. fermentum K11, L. fermentum K14, L. casei X11*	HT-29 cells	Anti-proliferation activity, Induction of apoptosis	Wang et al 2012
Research on animal models/*in vivo*				
1.	*Bacillus polyfermenticus*	Five-week-old male F344 rats	Reduced in the formation of aberrant crypt foci of about 50% and increased of antioxidant potential.	PARK et al. 2007
2.	*L. plantarum*	Six-month-old male and female Wistar albino rats	Reduced concentration of bile acid and bacterial enzymes. Increased level of TNF-alpha in the serum.	BERTKOVA et al. 2010
3.	*L. rhamnosus 231* (Lr231)	Male Wistar rats	Decreased fecal activity of azoreductase, nitroreductase, GST. Increased GSH.	GOSAI et al. 2011
4.	*L. acidophilus* KFRI342	Forty-five male F344 rats	Reduction in aberrant crypt foci, beta-glucuronidase, beta-glucosidase activity.	CHABG et al.

				2012
5.	*L. casei* BL23	C57BL/6 mice	Modulation of host immune response. *L.casei* BL23 protect mice against DMH-induced colorectal cancer.	LENOIR et al. 2016

Lactobacillus spp.

Lactobacillus spp. are one of lactic acid bacteria that are widely used and consumed probiotics in many commercial products for health modulation or treatment. They are facultative anaerobes that often grow better under microaerophilic conditions, Gram-positive non-spore-forming rods shape, non-motile, and catalase-negative (46). *Lactobacillus* spp. are members of the genus *Lactobacillus*, and family Lactobacillaceae. Their optimal growth temperature is 30-40°C, and pH of 5.5 – 5.8. They can grow at a pH lower than 5 (47). They produce lactic acid as the end-product of fermention divided into two groups. Homofermentative lactobacilli predominantly ferment sugars into lactic acid and do not produce gas, whereas heterofermentative lactobacilli produce lactic acid and other substances such as acetic acid, and CO_2 (48). *Lactobacillus* is composed of over 170 species and 17 subspecies found in a number of fermented food products and human. They inhabit in the gastrointestinal tract and vagina but can be opportunistic pathogens (46). In the gastrointestinal tract, there were many species of lactobacilli colonized including *L. fermentum, L. plantarum, L. casei, L. rhamnosus, L. antri, L. gastricus, L. kalixensis, L. reuteri, and L. ultunensis* (49).

Although the widely-used of probiotics are generally regarded as safe (GRAS) and beneficial for health, patients with compromised immune systems, leaky gut or critical illnesses should be aware of administering or consumtion of probiotics (36). Moreover, in general, probiotics has been suggested to consume approximately 10^9CFU per day based on the daily consumption of 100 g or mL of probiotic food (31). The aforementioned reports suggested that the consumption of probiotics with anticancer activities might prevent the development of CRC (50). However, the potential effects of probiotics depend on the strains of probiotics (51). The strain-specific phenomenon was found in a case-by-case manner, possibly due to differences in genetic background including ethnicity. Therefore, the benefit of each probiotic strain might not be generalizable to all population. In addition, only certain strains could colonize as long-term persistent microbiota in individual's gut (52).

Our previous studies demonstrated that *Lactobacillus* spp. isolated from feces of Thai infants and gastric biopsies of Thai patients had an anti-inflammatory effect by suppression of IL-8 production after *C. difficile* infection in HT-29 cells (9), inhibition of TNF production in LPS-activated THP-1 monocytoid cells, and inhibition of IL-8 production in *H. pylori*-stimulated AGS gastric epithelial cells (10). Anti-inflammatory effect is one of beneficial properties required for anticancer activity of probiotics (53). We have selected thirty-nine *Lactobaillus* strains with anti-inflammatory effect (Table 1 in Appendix B). We hypothesized that these probiotics might have other anticancer activities against CRC *in vitro*. Therefore, this study aimed to identify probiotic strains

with more anticancer activities against CRC cells. These probiotic strains may be useful for prevention or co-treatment in Thai patients with CRC in the future.

CHAPTER IV

MATERIAL AND METHODS

Bacterial cultures

Thirty-nine *Lactobacillus* strains tested are listed in Table 1. *Lactobacillus* spp. had previously been isolated from infant feces (9) and gastric biopsies of dyspeptic patients (10). All *Lactobacillus* strains were obtained from recent studies that approved by Ethics Committee for Human Research of Faculty of Medicine, Chulalongkorn University. All lactobacilli were thawed from the culture stock stored at the Department of Microbiology, Faculty of Medicine, Chulalongkorn University. All strains were cultured on deMan Rogosa Sharpe (MRS) agar in an anaerobic chamber (Concept Plus, Ruskinn Technology, UK) with 10% CO2, 10% H2, and 80% N2, at 37°C for 24 h. Bacterial stock cultures were stored at -80°C in MRS broth (Oxoid, Hampshire, UK) containing 20% (v/v) glycerol.

Table 4 *Lactobacillus* strains tested

No.	*Lactobacillus* species	Strains	Origins
1	*Lactobacillus gasseri*	L2	Infant feces
2	*Lactobacillus gasseri*	L3	Infant feces
3	*Lactobacillus salivarius*	L8	Infant feces
4	*Lactobacillus gasseri*	L10	Infant feces
5	*Lactobacillus salivarius*	L11	Infant feces
6	*Lactobacillus salivarius*	L17	Infant feces
7	*Lactobacillus vaginalis*	L19	Infant feces
8	*Lactobacillus gasseri*	L20	Infant feces
9	*Lactobacillus salivarius*	L22	Infant feces
10	*Lactobacillus salivarius*	L23	Infant feces

11	*Lactobacillus gasseri*	L26	Infant feces
12	*Lactobacillus gasseri*	L29	Infant feces
13	*Lactobacillus gasseri*	L30	Infant feces
14	*Lactobacillus gasseri*	L32	Infant feces
15	*Lactobacillus rhamnosus*	L33	Infant feces
16	*Lactobacillus rhamnosus*	L35	Infant feces
17	*Lactobacillus casei group*	B13	Human gastric biopsy
18	*Lactobacillus gasseri*	XB68	Human gastric biopsy
19	*Lactobacillus plantarum*	B90	Human gastric biopsy
20	*Lactobacillus gasseri*	XB94	Human gastric biopsy
21	*Lactobacillus salivarius*	B101	Human gastric biopsy
22	*Lactobacillus casei group*	B106	Human gastric biopsy
23	*Lactobacillus casei group*	B107	Human gastric biopsy
24	*Lactobacillus salivarius*	B109	Human gastric biopsy
25	*Lactobacillus plantarum*	B6	Human gastric biopsy
26	*Lactobacillus plantarum*	B7	Human gastric biopsy
27	*Lactobacillus salivarius*	B8	Human gastric biopsy
28	*Lactobacillus salivarius*	B21	Human gastric biopsy
29	*Lactobacillus gasseri*	XB41	Human gastric biopsy
30	*Lactobacillus salivarius*	B47	Human gastric biopsy
31	*Lactobacillus gasseri*	XB48	Human gastric biopsy
32	*Lactobacillus gasseri*	XB58	Human gastric biopsy
33	*Lactobacillus plantarum*	B67	Human gastric biopsy
34	*Lactobacillus plantarum*	B70	Human gastric biopsy
35	*Lactobacillus salivarius*	B73	Human gastric biopsy
36	*Lactobacillus salivarius*	B74	Human gastric biopsy
37	*Lactobacillus gasseri*	XB95	Human gastric biopsy
38	*Lactobacillus gasseri*	XB96	Human gastric biopsy
39	*Lactobacillus salivarius*	B52	Human gastric biopsy
40	*Lactobacillus rhamnosus*	GG	ATCC 53103

Cell line culture

The human colon adenocarcinoma cell line Caco-2 and HT-29 cells were originally obtained from the American Type Culture Collection (ATCC). Caco-2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS; Gibco-Invitrogen, USA), 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; (Gibco-Invitrogen, USA), and 1% sodium pyruvate (Gibco-Invitrogen, USA). HT-29 cells were maintained in DMEM supplemented with 10% heat-inactivated FBS, and 1% (w/v) penicillin and streptomycin (Gibco-Invitrogen, USA). The cells were incubated at 37°C in a humidified 5% CO_2 incubator (54, 55).

Preparation of Lactobacillus conditioned media (LCM)

Lactobacillus isolates were cultured anaerobically in MRS medium at 37°C for 24-48 h in an anaerobic chamber (Concept Plus). A single colony of Lactobacillus was re-streaked and grown on MRS agar for 24 h. After incubation, a single colony was inoculated in 5 ml of MRS broth and grown at 37°C for 24 h. The culture was adjusted to an OD600 nm of 0.1 in a total volume of 10 ml and then incubated under anaerobic conditions for 24 h. The culture media which were collected by centrifugation at 4000 ×g for 10 min at 4°C, filter-sterilized using a 0.22-μm syringe filter (Acrodisc, US) and concentrated by speed-vacuum drying (Speed vacuum DNA 110, Savant, NY) is called as “Lactobacillus conditioned medium (LCM)”. In addition, LCM in this experiment was and was not pH adjusted to pH 7.6 to determine the

effect of pH on cell proliferation. The dried pellet was resuspended in 500 µl of cell line culture media and stored at −20°C until use (56).

Cell proliferation assay

MTS assay

Cell viability of human cancer cell lines (Caco-2 cells) was determined by using MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay: Promega, Madison, WI) following the manufacturer's instructions. In brief, seeding the cells at the 6.0×10^3 cells per well (100µl/well) into 96-well plates and incubated overnight for attachment. After that, replaced the culture media by fresh medium containing the different concentrations of LCM. The treatment incubated for 24, 48, and 72 h. MTS assay was carried out using MTS reagent: DMEM sample at a ratio of 1:5 was added and incubated for an additional 2 h under the same conditions. The optical density of formazan demonstrating cell viability measured at 490 nm using a microplate reader (Varioskan Flash, Thermo Scientific, USA). All assays performed in triplicates and three times independent experiments. Cells incubated with MRS alone were used as controls (57, 58).

The percentage of proliferation cells was calculated by the following formula:

Proliferation of cells (%) = (ODsample - OD medium)/ ODcontrol - ODmedium) × 100

MTT assay

Cell viability was determined using MTT (2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide; Abcam, UK) assay following the manufacturer's

instructions. In brief, HT-29 and Caco-2 cells were seeded at the 5.0-6.0 × 10^3 cells per well (100 µl/well) into 96-well plates and stabilized for 24 h (37°C, 5% CO_2) for cell attachment. After that, the culture media was completely replaced by a fresh medium containing LCM. The treatment was incubated for 24, 48, and 72 h. After incubation, 15 µl of Phosphate-buffered saline (PBS) containing 5 mg/ml MTT reagent was added to each well and the plates were further incubated for 3 h. Then, 150 µl of Dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) was added to dissolve formazan crystals. The optical density of formazan demonstrating cell viability was measured at 570 nm using a microplate reader (Varioskan Flash, Thermo Scientific, USA). Cells incubated with MRS alone were used as controls (59, 60). The percentage of proliferation cells was calculated by the above formula from MTS assay. All assays were performed in triplicates and three times independent experiments.

Flow cytometry

Cell viability was measured by 7-amino-actinomycin D Viability Staining Solution (7-AAD; BioLegend) according to the manufacturer's instructions. In this assay, CRC cells were seeded into 24-well plates at a density of 100,000 cells per well for 24 h. The cells were then incubated with lactobacilli at a different Multiplicity of infection (MOI) in each well for 24, and 48. Afterward, the cells were harvested with 0.25% Trypsin-EDTA (Gibco, USA) and rinsed 3 times with sterile PBS. After centrifugation, the cell pellets were re-suspended in 50 µl Flow Cytometry Staining Buffer (FACS buffer) that containing 1 µl of 7-AAD and incubated for 10 min

under light protection before analysis. The cells were analyzed by using CytoFLEX Flow Cytometer (Beckman Coulter, US). The untreated cells were used as control. All data were analyzed using FlowJo software (61, 62).

Adhesion assay

Caco-2 cells were seeded into 24-well plates at a density of 250,000 cells per well and the medium was replaced every two days for two weeks to obtain differentiated cells (63). After that, lactobacilli were grown in MRS broth for 24 h and harvested by centrifugation at 4000 ×g for 10 min at 4 °C. After centrifugation, the pellet was washed with sterile PBS and re-suspended in the complete medium. Before adding lactobacilli cell suspension, the cell monolayer was washed with sterile PBS to remove antibiotics. Lactobacilli cell suspension (MOI = 100, 1,000) was added to the wells and synchronized at 600 ×g for 2 min. After 2 h incubation, the cell monolayer was washed three times with sterile PBS to remove unbounded bacteria and detached with Triton X-100 (0.05%). The adherent lactobacilli number was determined by the plate count method on MRS agar. The plates were incubated for 24-48 h at 37°C and the colony-forming units (CFU mL^{-1}) were counted.

The percentage of adherent lactobacilli was calculated by the following formula (64-66):

$$\text{adhesion (\%)} = (CFU_{end}/CFU_{initial}) \times 100$$

Scanning electron microscopy (SEM)

SEM was employed to confirm polarized cells for adhesion assay. Caco-2 cells were seeded into 12-well plates at a density of 500,000 cells per well and the medium was replaced every two days for two weeks. Lactobacilli cell suspension was added to the wells and synchronized at 600 ×g for 2 min. After 2 h incubation, the cell monolayer was washed three times with sterile PBS to remove unbounded bacteria. Samples were sent for coating and analysis at Scientific and Technological Research Equipment Centre Chulalongkorn University (STREC). Firstly, samples were primarily fixed with 2.5% w/v glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h. Then, the samples were washed twice with PBS and distilled water (DW) with each step 5 min. Samples were dehydrated in an increasing series of Ethanol, starting with 30%, followed by 50%, 70%, 95% with each step 10 min, thereafter 100% ethanol, 3 times 5 min. Finally, cells were dried in a critical point dryer (Leica model EM CPD300, Austria) and coated with gold on a sputter coater (Balzers model SCD040, Germany). The samples were examined using SEM (JSM- IT500HR, JEOL, Japan) (66).

Statistical Analysis

All experiments were performed in triplicate for each condition and repeated three times for independent experiments. The data were shown as mean ± standard deviation (SD). The data were analyzed using Graphpad ver. 8.4.3 (686). Statistical analysis to compare among groups were performed by two–way analysis of variance (ANOVA). A difference at $P < 0.05$ was considered statistically significant.

CHAPTER V

RESULTS

This study aimed to identify new *Lactobacillus* strains with anticancer activities against colorectal cancer. All 39 lactobacilli used in this study were shown previously to have anti-inflammatory activity, for example, suppression of IL- 8 secretion from HT-29 cells induced by *C. difficile* infection, inhibition of TNF production in LPS-activated THP-1 monocytoid cells, and inhibition of IL-8 production in *H. pylori*-stimulated AGS gastric epithelial cells (unpublished data, Table 1 in appendix B).

Part I. Optimization of LCM for anti-proliferative activity against CRC cells

LCM from *Lactobacillus gasseri* strain L2 and *L. gasseri* strain L3 was used to determine optimal number of CRC cells to be used for anti-proliferative activity. Firstly, the cell number of representatives of CRC cell lines, HT-29 cells, and time point was determined (Figure 8A). HT-29 cells were seeded at the density of 3×10^4 cells/ml (3×10^3 cells/well) and 5×10^4 cells/ml (5×10^3 cells/well) and grown for 24, 48, and 72 h. The results showed that HT-29 cells at both seeding cell numbers could grow continuously over time points up to 72 h. Therefore, the optimal time point to use CRC cells could be up to 72 h in this experiment. Next, the optimal seeding cell number at the concentration of 3×10^4 cells/ml (Figure 8B) and 5×10^4 cells/ml (Figure 8C) to be used with pH unadjusted and pH adjusted LCM from *Lactobacillus gasseri* strain L2 and *L. gasseri* strain L3 was determined for anti-

proliferative activity. The seeding cell density of 5 × 10^4 cells/ml showed different cell viability between various concentrations of LCM (Figure 7C). Therefore, HT-29 cells at the seeding number of 5 × 10^4 cells/ml was subsequently used to determine anti-proliferative effect of LCM.

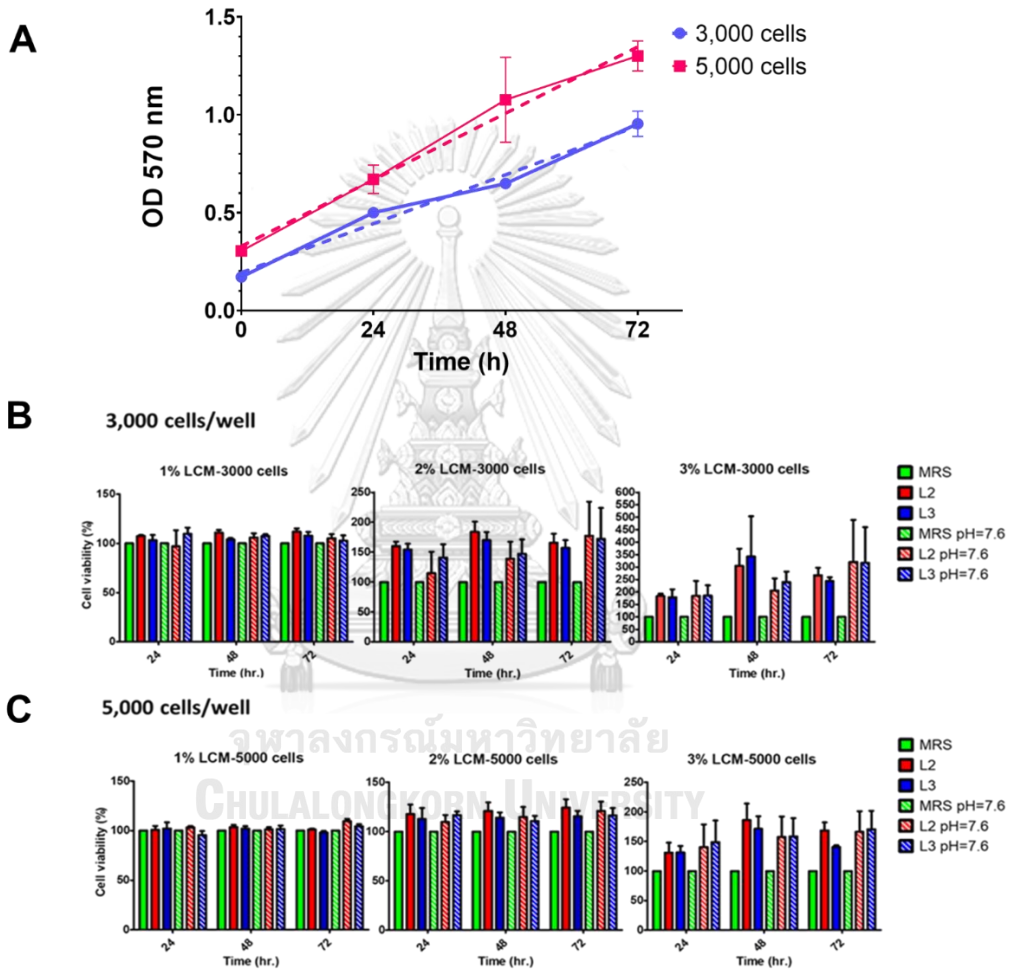


Figure 8 The growth curve of different seeding cell numbers of HT-29 cells at 24, 48, and 72 (A). The effect of different concentrations of LCM on viability of HT-29 cells at the cell density of 3 × 10^4 cells/ml (3 × 10^3 cells/well) (B) and 5 × 10^4 cells/ml (5 × 10^3 cells/well) (C) for 24, 48, and 72 h. This experiment was measured by MTT assay. All data are from 3 independent experiments. Data are presented as mean ± SD (n=3).

Likewise, in Caco-2 cells, the seeding cell density of 6×10^4 cells/ml (6×10^3 cells/well) was selected to be used to determine anti-proliferative effect of LCM (Data not shown).

Viability test used to determine anti-proliferative effect on CRC cell lines

To determine the anti-proliferation effect of LCM on CRC cells, this experiment used MTS assay to detect cell viability. The optimization of the optimal condition for this experiment started with the different concentrations of the MRS medium alone. I optimized by adding the concentration of 5%, 10%, 20%, and 40% (v/v) of MRS alone incubated with Caco-2 cells at 24 h. After that, the cell viability was measured by MTS assay. The result was found unrelated results between cell viability by measuring the OD value and morphology under the light microscopic. At the concentration of 40% (v/v) of MRS (Figure 9E), the cell morphology in the well was changed compared with normal Caco-2 cell (Figure 9F). The cells were found dead contrary to the OD 490 nm value showed an increase in cell viability when compared with normal cells. Unrelated OD 490 nm value by MTS assay might be affected in the calculation of the percentage of cell viability. LCM which was originally the yellow-brown color from MRS, and different pH related to each strain. So, the complete medium was varied between each sample. This variation affected the measurement of Absorption. The cell viability from the MTS assay was varied. Thus, I decided to change the method to use the MTT assay for detecting cell viability. MTT was one method used for detecting cell viability in vitro. The difference

between MTS and MTT assay was the formazan product solubilization step. The formazan product in the MTS assay is soluble, so the MTS assay did not need a step to solubilize the formazan. However, after incubated with MTT reagent, changed the old medium and solubilized the formazan product with DMSO instead. Thus, the MTS assay was not suitable for my experiment because the different colors in each well may interfered with OD detection.

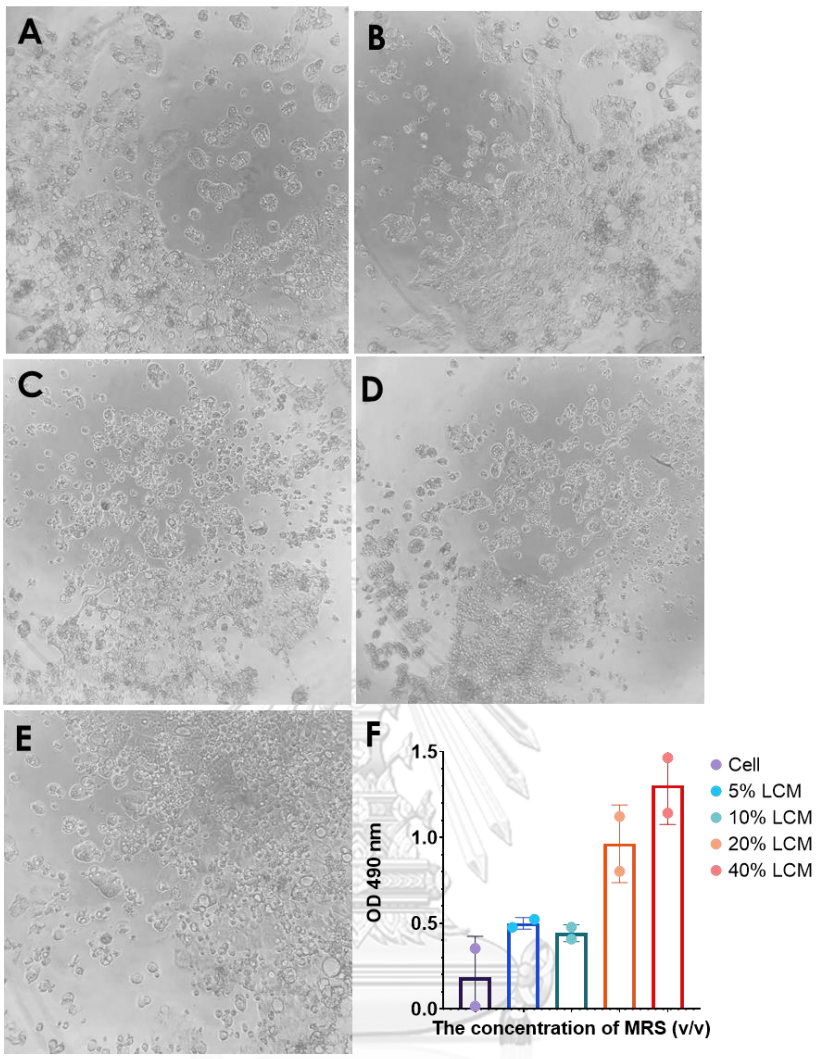


Figure 9 Morphology of Caco-2 cell lines after treated with MRS with the concentrations of 5% (v/v) (A), 10% (v/v) (B), 20% (v/v) (C), and 40% (v/v) (D) of MRS medium alone and normal Caco-2 cell (E) at 24 h (Magnification x 100). The effect of different concentrations of MRS medium on viability of Caco-2 cells at 24 h (f).

The OD 490 nm value was measured by using MTS assay to detect cell proliferation. All data are representative of 2 independent experiments. Data are presented as mean ± SD (n=2).

Effect of MRS medium on viability of CRC cell lines

HT-29 and Caco-2 cell lines were treated with different concentrations of MRS medium to determine the cytotoxic effect before LCM screening because MRS medium that used as a control in this experiment.

In HT-29 cells, the result found that HT-29 cells were treated with LCM less than 3% (v/v) of LCM (Figure 10A). The morphology of HT-29 cells under the light microscope was changed after treated with 3% (v/v) of MRS until 24 h. The cell morphology under the light microscope shown partly cell shrinkage and cell death property. However, the dead cell numbers were related to MTT results (Figure 10B). The median of the OD values was much lower than the HT-29 cells without any treatment. It was likely that the concentration of LCM used in this experiment should be less than 3% (v/v). Thus, two concentrations that were chosen to see the dose-dependent was the concentration of 1% and 2% (v/v).

Also, in Caco-2 cells, I optimized the same method as HT-29 cells. According to the preliminary result from the MTS assay, I found the range of concentration of LCM that could be used in this experiment related to the cell morphology under the light microscopic. This optimization of Caco-2 cells varied the concentration of LCM that was 2% (v/v), 4% (v/v), and 8% (v/v) for covering the concentration of 5% (v/v) (Figure 1 In Appendix B). The result showed the optimal LCM for used should be below 4% (v/v) of LCM. So, I chose the acceptable concentration range that was 1% (v/v), 2% (v/v), and 4% (v/v) to see the dose-dependent effect of LCM (Figure 11).

There was a two-fold increase in a concentration that might show the expected dose-dependent manner.

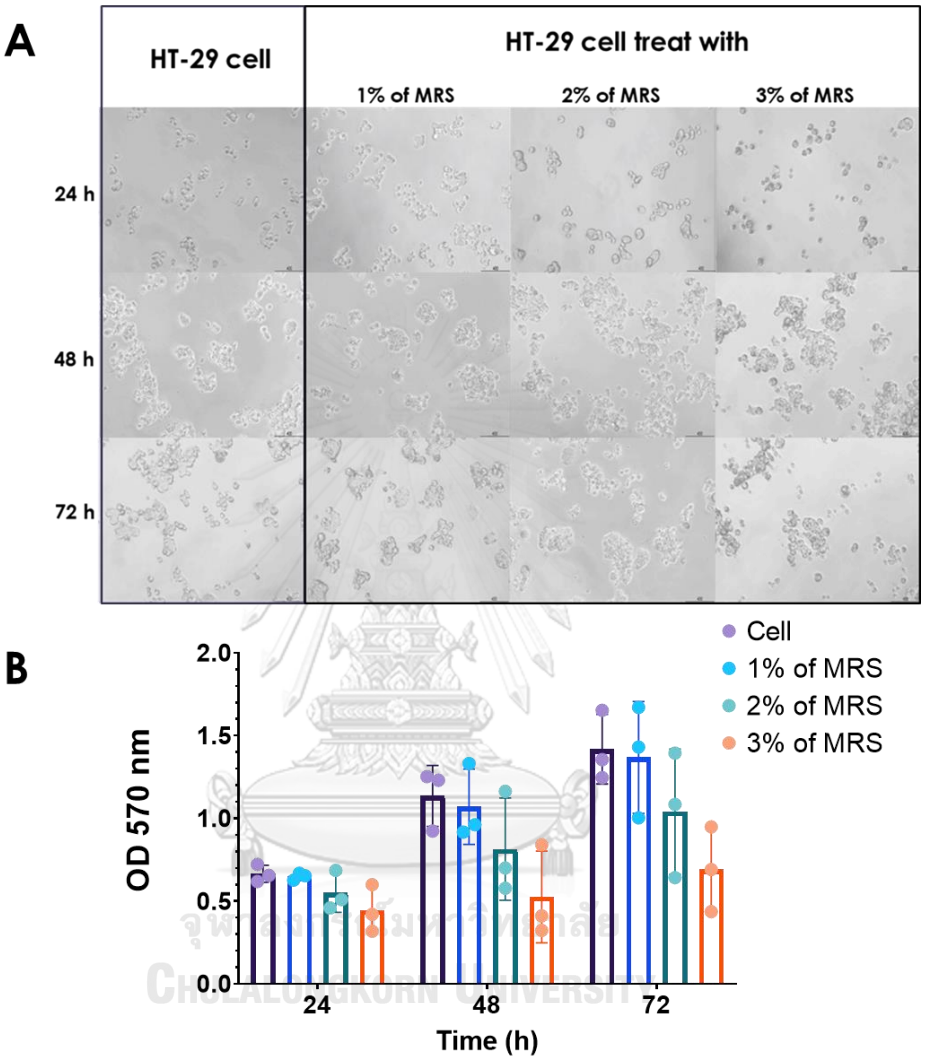


Figure 10 Morphology of HT-29 cell lines after treated with different concentrations of MRS medium alone at 24, 48 and 72 h (Magnification x 100) (A). The effect of different concentrations of MRS medium on viability of HT-29 cell lines at 24, 48 and 72 h (B).

The OD 570 nm value was measured by using MTT assay to detect cell proliferation. All data are representative of 3 independent experiments. Data are presented as mean ± SD (n=3).

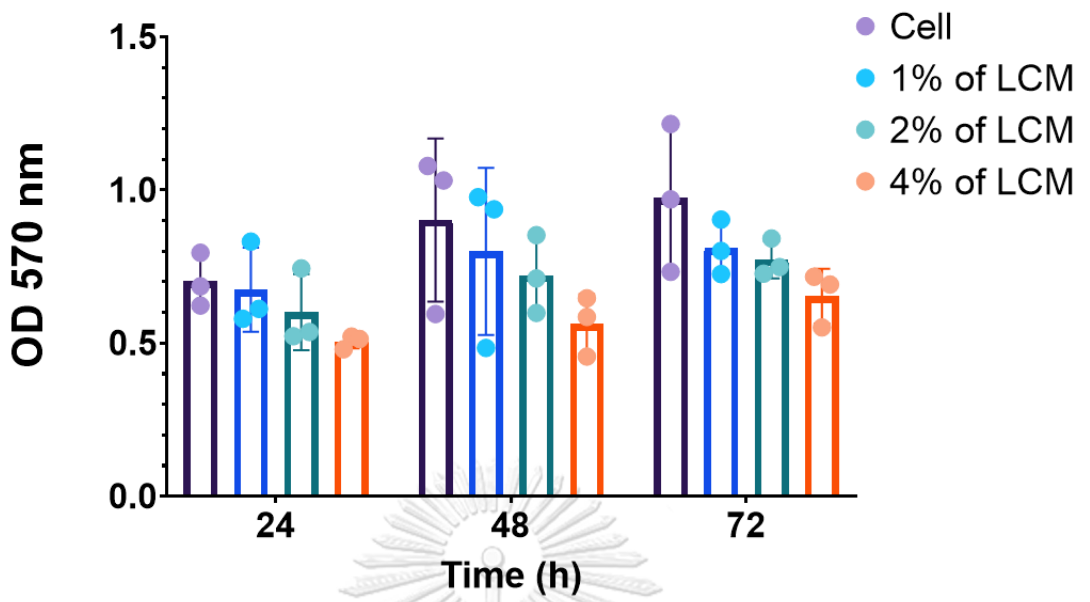


Figure 11 The effect of different concentrations of MRS medium on viability of Caco-2 cell lines at 24, 48 and 72 h.

The OD 570 nm value was measured by using MTT assay to detect cell proliferation. All data are representative of 3 independent experiments. Data are presented as mean ± SD (n=3).

Part II. Anti-proliferative effect of *Lactobacillus* cultured medium (LCM) on CRC cell lines

To determine the effect of secreted metabolites of probiotics on viability of CRC cells, LCM from 39 different *Lactobacillus* strains were tested with two representatives of CRC cell lines, HT- 29 and Caco -2 cells. In addition, LCM used in this experiment was 1) pH unadjusted (pH 2-4) and 2) pH adjusted to 7.6 to determine the effect of pH on cell viability.

Anti-proliferative effect of LCM on HT-29 cell line

HT-29 cells were treated with LCM from each *Lactobacillus* strain at the concentration of 1% and 2% for 24, 48, and 72 h and the cell viability were measured by MTT assay (Figure 2 in Appendix B). The results showed that LCM from 11 out of 39 *Lactobacillus* strains slightly reduced cell viability of HT-29 cell line. However, LCM derived from 2 *Lactobacilli* strains, *L. vaginalis* strain L19 and *L. gasseri* strain L20, demonstrated strongest anti-proliferative effect against HT-29 cells (Figure 12). 1% pH unadjusted LCM from *L. vaginalis* L19 and *L. gasseri* L20 seemed to reduce cell viability, compared with MRS control, whereas other strains such as *L. casei* group B107 slightly inhibited cell growth (Figure 12A). At 24 h incubation, LCM from *L. vaginalis* L19 and *L. gasseri* L20 decreased cell viability to 81.00 ± 17.78 % and 88.77% ± 21.64 %, respectively, compared with the MRS control. At 48 h incubation, the cell viability of HT-29 cells was reduced to 84.39 ± 17.74 % and 93.13 ± 14.69 %, respectively. At 72 h incubation, the cell viability was 74.42 ± 11.60 %

and 93.47 ± 8.97 %, respectively. However, the reduction of HT-29 cell viability after treatment with 1% LCM from these 2 strains was not statistically different from that after treatment with the MRS control.

pH unadjusted LCM from *L. vaginalis* L19 and *L. gasseri* L20 at the concentration of 2% demonstrated the reduction of the cell viability when compared with the MRS control (Figure 12B). At 24 h incubation, only LCM from *L. vaginalis* L19 suppressed the cell growth to 86.31 ± 13.36%. At the 48-h incubation, LCM from *L. vaginalis* L19 significantly suppressed the cell growth to 91.49 ± 0.38 % ($p < 0.001$), compared with the control, whereas LCM from *L. gasseri* L20 did not showed significantly reduced. Moreover, at the 72 h of incubation, LCM from *L. gasseri* L20 significantly reduced cell viability to 88.87 ± 2.05% ($p < 0.05$).

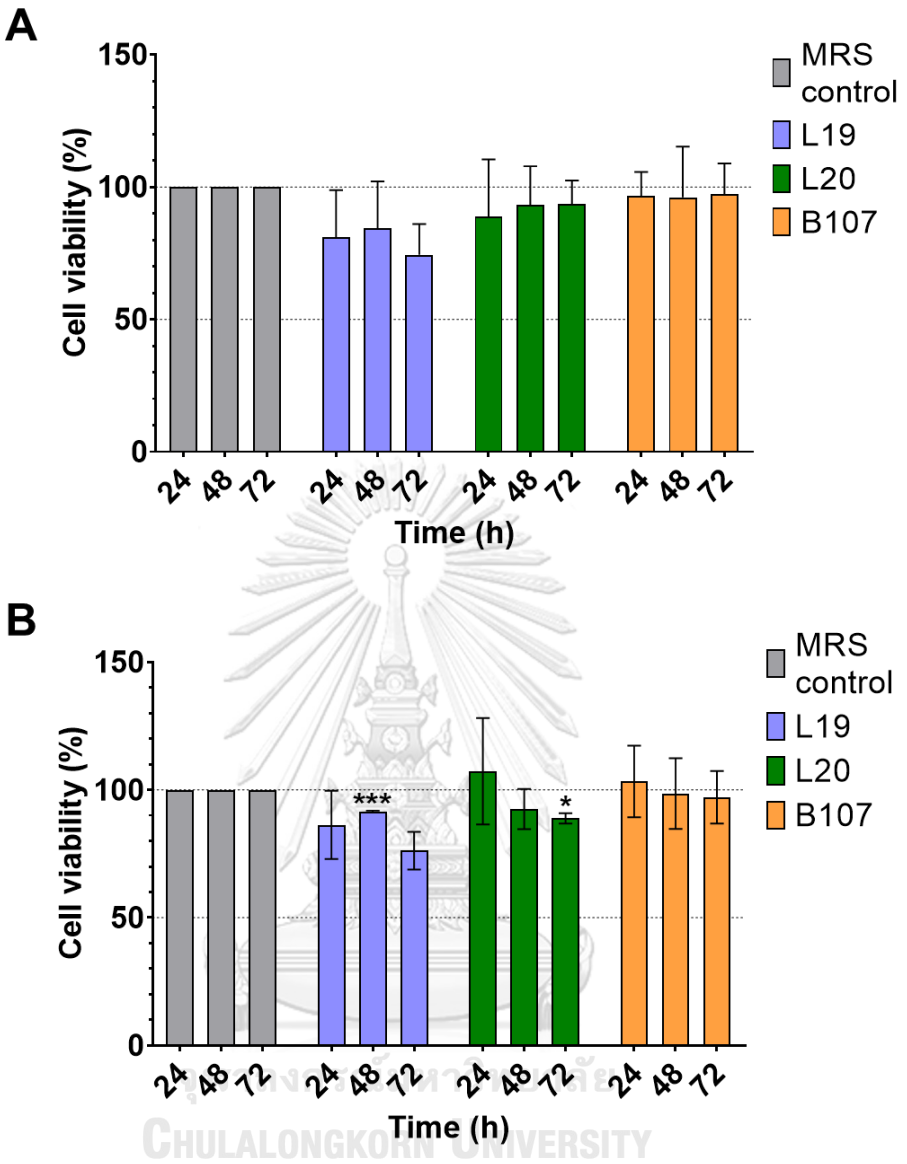


Figure 12 Anti-proliferative effect of pH unadjusted *Lactobacillus* condition medium (LCM, pH 2-4) at the concentration of 1% (v/v) (A) and 2% (v/v) (B) on the viability of HT-29 cells at 24, 48, and 72 h incubation.

All data are the results from 3 independent experiments. Data are presented as mean ± SD (n=3), Differences between MRS control and treated groups are considered statistically significant when $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). L19: *L. vaginalis* L19; L20: *L. gasseri* L20; B107: *L. casei* group B107

Anti-proliferative effect of pH adjusted LCM on HT-29 cell line

To investigate whether acidic pH or secreted metabolites of LCM resulted in anti-proliferative effect on HT-29 cell line, pH of LCM was adjusted to 7.6 that mentioned as the pH of DMEM (cell culture medium) and physiologic pH in human lumen before use. The effect of pH adjusted LCM was shown in figure 13. At the concentration of 1%, pH adjusted LCM from *L. vaginalis* L19 showed slight suppression of the cell growth at 24 and 48 h incubation to 96.61 ± 11.34% and 95.28 ± 5.17%, respectively. In contrast, at 72-h incubation, the cells grew to 108.46 ± 10.88%. However, the LCM of *L. gasseri* L20 seemed to suppress the cell viability at 24, 48, and 72 h to 91.36 ± 13.07%, 93.48 ± 18.16%, and 87.70 ± 10.87%, respectively (Figure 13A). In addition, the cell viability was slightly decreased with no statistical difference after 24 h incubation with 2% pH adjusted LCM from *L. vaginalis L19* and *L. gasseri* L20 was likely to suppress cell growth at 24 h incubation (Figure 13B).

According to the results of anti-proliferative activity screening on HT-29 cells, LCM obtained from *L. vaginalis* L19 and *L. gasseri* L20 significantly reduced cell viability more than other strains. The significant reduction of cell viability was concluded in Table 5. Therefore, both strains were further examined for their anti-proliferative effect on Caco-2 cell line.

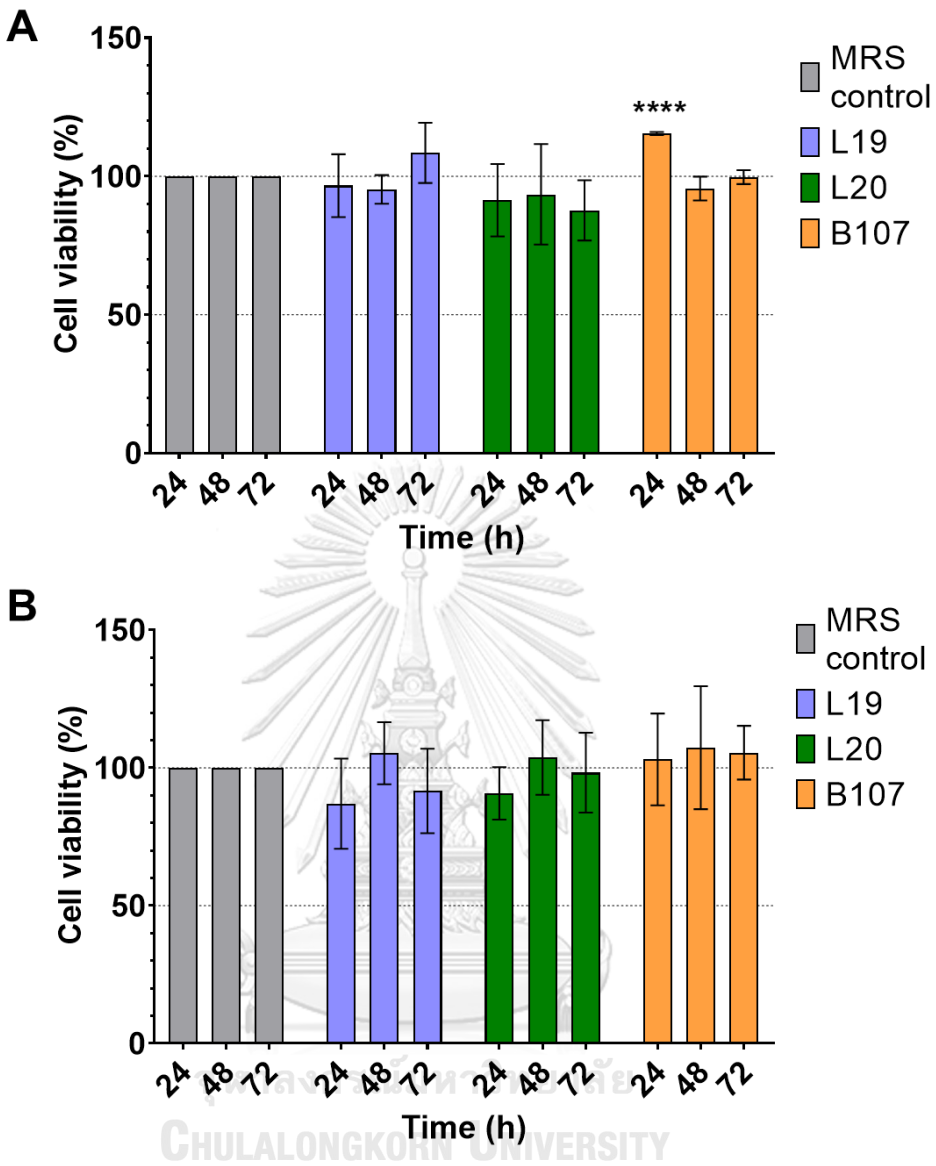


Figure 13 Anti-proliferative effect of pH adjusted *Lactobacillus* conditioned media (LCM, pH adjusted to 7.6) at the concentration of 1% (v/v) (A) and 2% (v/v) (B) on the viability of HT-29 cells at 24, 48, and 72 h incubation.

All data are the results from 3 independent experiments. Data are presented as mean ± SD (n=3). The difference between the MRS control and treated groups is considered statistically significant when $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). L19: *L. vaginalis* L19; L20: *L. gasseri* L20; B107: *L. casei* group B107

Anti-proliferative effect of pH unadjusted LCM on Caco-2 cell line

Based on the preliminary results of the effect of MRS alone on Caco-2 cell viability (Figure 11), this experiment used the concentration of 1, 2, and 4% LCM to examine the effect of secreted metabolites of *L. vaginalis* L19 and *L. gasseri* L20 on viability of Caco-2 cells.This experiment also compared unadjusted and pH adjusted LCM to determine whether acidic pH play a role in the anti-proliferative effect against Caco-2 cells.

Anti-proliferative effect of pH adjusted LCM on Caco-2 cell line

To examine the anti-proliferative effect on Caco-2 cells, the concentrations of LCM used in this experiment were 1, 2, and 4%.

The result showed that pH unadjusted LCM of *L. vaginalis* L19 at all 3 concentrations did not suppress Caco-2 cell growth (Figure 14). Although 4% LCM from *L. gasseri* L20 seemed to slightly reduce the cell viability after 48 h incubation, it was not statistically significant difference from the MRS control (Figure 14C).

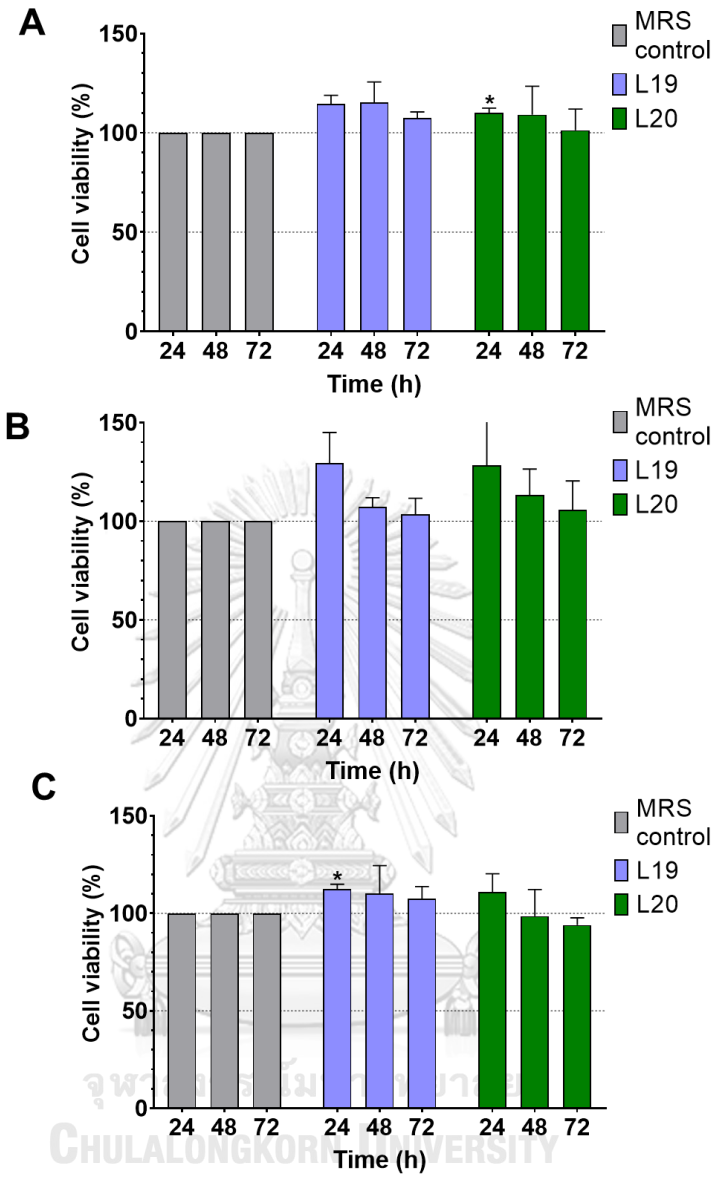


Figure 14 Anti-proliferation effect of pH unadjusted *Lactobacillus* conditioned media (LCM, pH 2-4) at the concentration 1% (v/v) (A), 2% (v/v) (B), and 4% (v/v) on the viability of Caco-2 cells at 24, 48, and 72 h incubation.

All data are the results from 3 independent experiments. Data are presented as mean ± SD (n=3). The difference between the MRS control and treated groups is considered statistically significant when $p < 0.05$ (*). L19: *L. vaginalis* L19; L20: *L. gasseri* L20.

In contrast to HT-29 cells, viability of Caco-2 cells was higher reduced by LCM from *L. gasseri* L20 when pH was adjusted to 7.6 at all 3 concentrations. At 24 h incubation, the result showed significant reduction at 4% LCM. The cell viability was 91.76 ± 1.64% (Figure 15 C).

In summary, the anti-proliferative activity of LCM on CRC cells showed that LCM from 2 out of 39 strains of *Lactobacillus* spp., *L. vaginalis* L19 and *L. gasseri* L20, exhibited a significant reduction of HT-29 cell viability. LCM of *L. vaginalis* L19 did not affect the cell viability of Caco-2 cells, whereas LCM of *L. gasseri* L20 showed mild anti-proliferative effect. However, the pH adjusted LCM of *L gasseri* L20 also reduced the cell viability. The significant reduction was concluded in Table 5

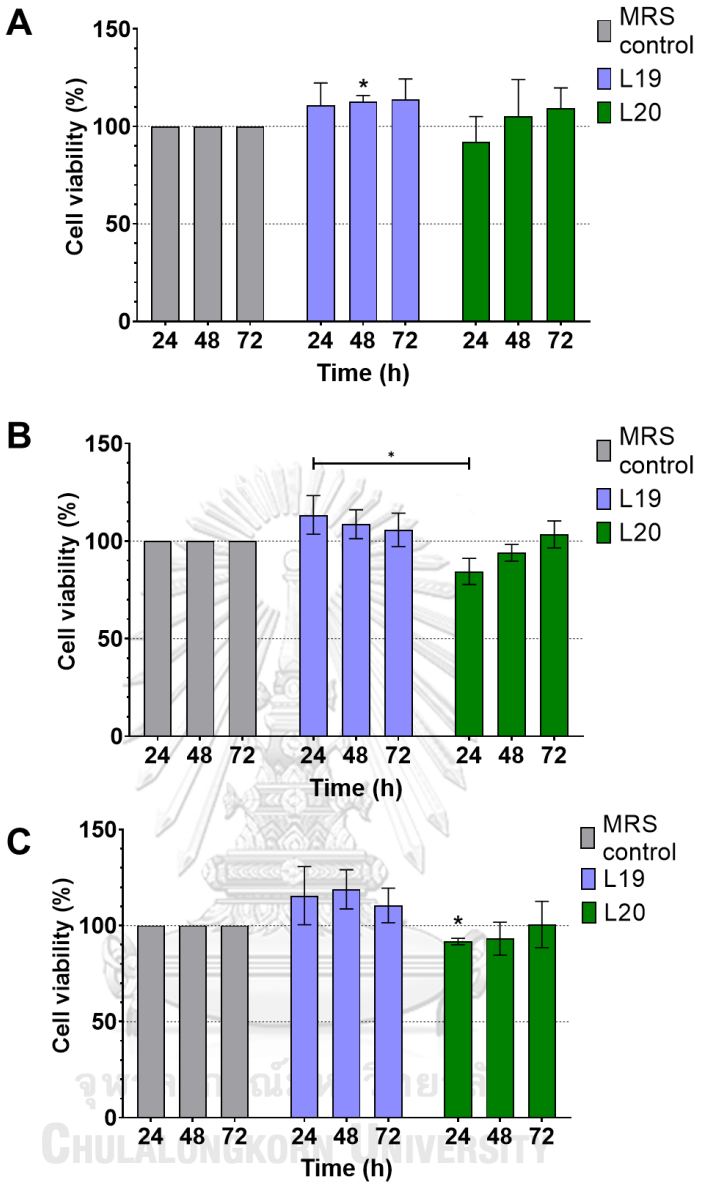


Figure 15 Anti-proliferation effect of pH adjusted *Lactobacillus* conditioned media (LCM, pH adjusted to 7.6) at the concentration of 1% (v/v) (A), 2% (v/v) (B), and 4% (v/v) (C) on the viability of Caco-2 cells at 24, 48, and 72 h incubation.

All data are the results from 3 independent experiments. Data are presented as mean ± SD (n=3). The different between the MRS control and treated groups is considered statistically significant when $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). L19: *L. vaginalis* L19; L20: *L. gasseri* L20.

Lactobacilli Strains	The concentration of LCM (%)	Anti-proliferation activity											
		HT-29 cells						Caco-2 cells					
		pH unadjusted			pH adjusted			pH unadjusted			pH adjusted		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
L. vaginalis L19	1%	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	NS
	2%	NS	***	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	4%	-	-	-	-	-	-	*	NS	NS	NS	NS	NS
L. gasseri L20	1%	NS	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	NS
	2%	NS	NS	*	NS	NS	NS	NS	NS	NS	NS	NS	NS
	4%	-	-	-	-	-	-	NS	NS	NS	*	NS	NS

Table 5 The antiproliferative effect of *Lactobacillus* conditioned media (LCM) from *L. vaginalis* L19 and *L. gasseri* L20 on HT-29 and Caco-2 cells.

***** represents the statistically significant difference between the MRS control and treated groups when $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). NS indicated no significant difference between control and treated groups. Red font indicated no effect.

Part III. Anti-proliferative effect of viable lactobacilli on CRC cell lines

In the previous experiments in Part II, LCM showed weak anti-proliferative activity on CRC cell lines. To function as probiotics, lactobacilli not only produce metabolites secreted in LCM but also contain other beneficial compositions. Therefore, intact and viable lactobacilli were further used to determine anti-proliferative effect on CRC cells.

MTT assay was initially used to examine the anti-proliferative effect of viable lactobacilli against Caco-2 cells. After incubation with Caco-2 cells, lactobacilli were removed before measuring the cell viability. However, the OD measurement in each well was not correlated with viability of Caco-2 cells examined under a microscope (Figure 10 Appendix B). It is possible that lactobacilli were not completely removed from each well despite several washing steps and remained in a sufficient number to interfere with the MTT assay. These results indicated that the MTT assay was not appropriate to determine cytotoxic effect of lactobacilli. Therefore, flow cytometry was alternatively used to determine anti-proliferative effect of viable lactobacilli on CRC cells.

Anti-proliferative effect of viable lactobacilli on Caco-2 cells

Live lactobacilli at different MOIs were co-incubated with Caco-2 cells for 24 and 48 h. After live lactobacilli were incubated with Caco-2 cells at the MOI of 100 and 1000, morphological changes including cell shrinkage or cell death were observed at 24 h (Figure 16) and 48 h (Figure 17) compared with uninfected Caco-2 cells. *Lactobacillus rhamnosus* GG (ATCC 53103) was used as a positive control in this experiment because previous studies showed strong cytotoxic effect against many CRC cell types including Caco-2 cells (67).

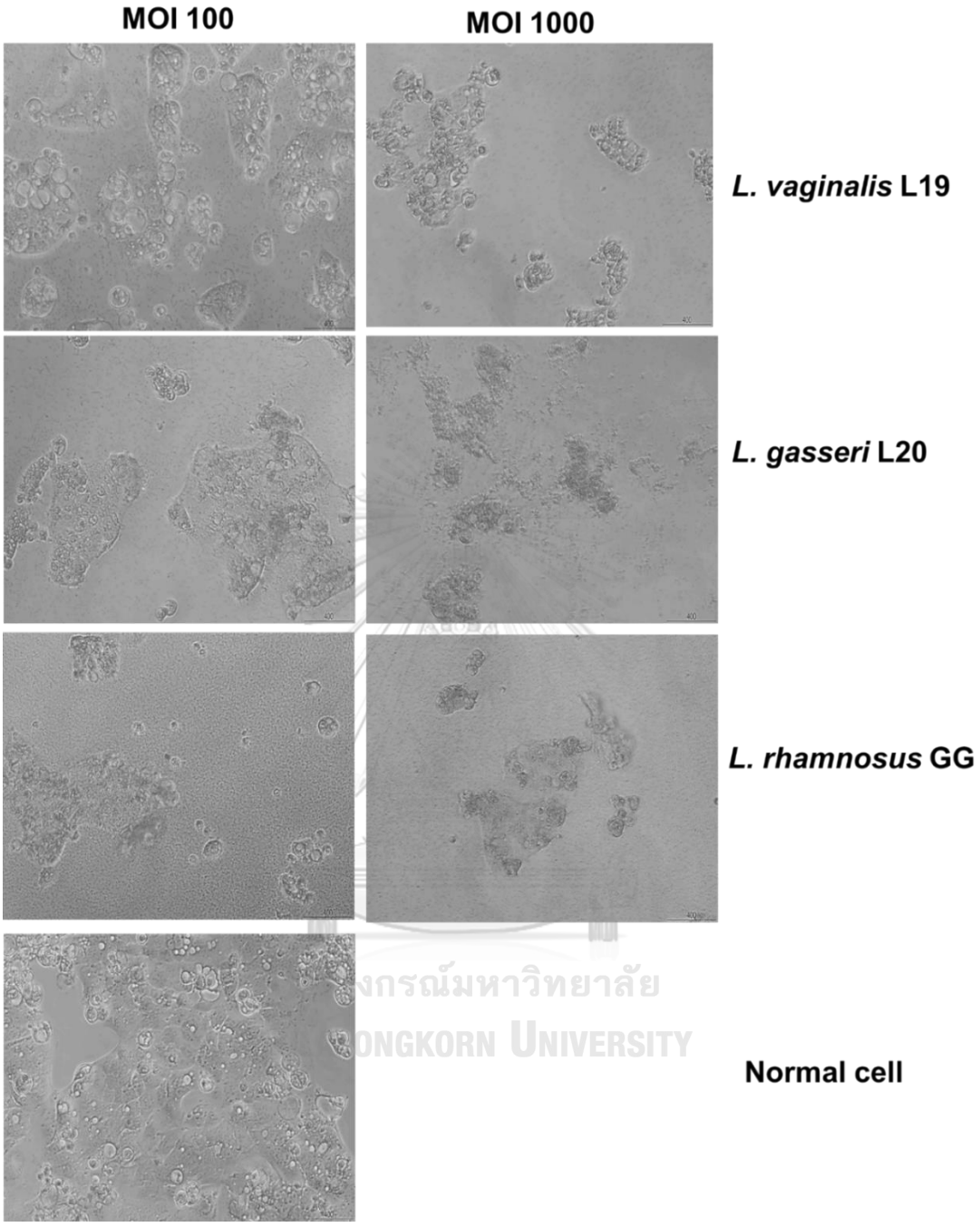


Figure 16 Morphology of Caco-2 cells after incubation with lactobacilli at the MOI of 100 and 1000 for 24 h (Magnification × 100).

Normal cell is uninfected cell control.

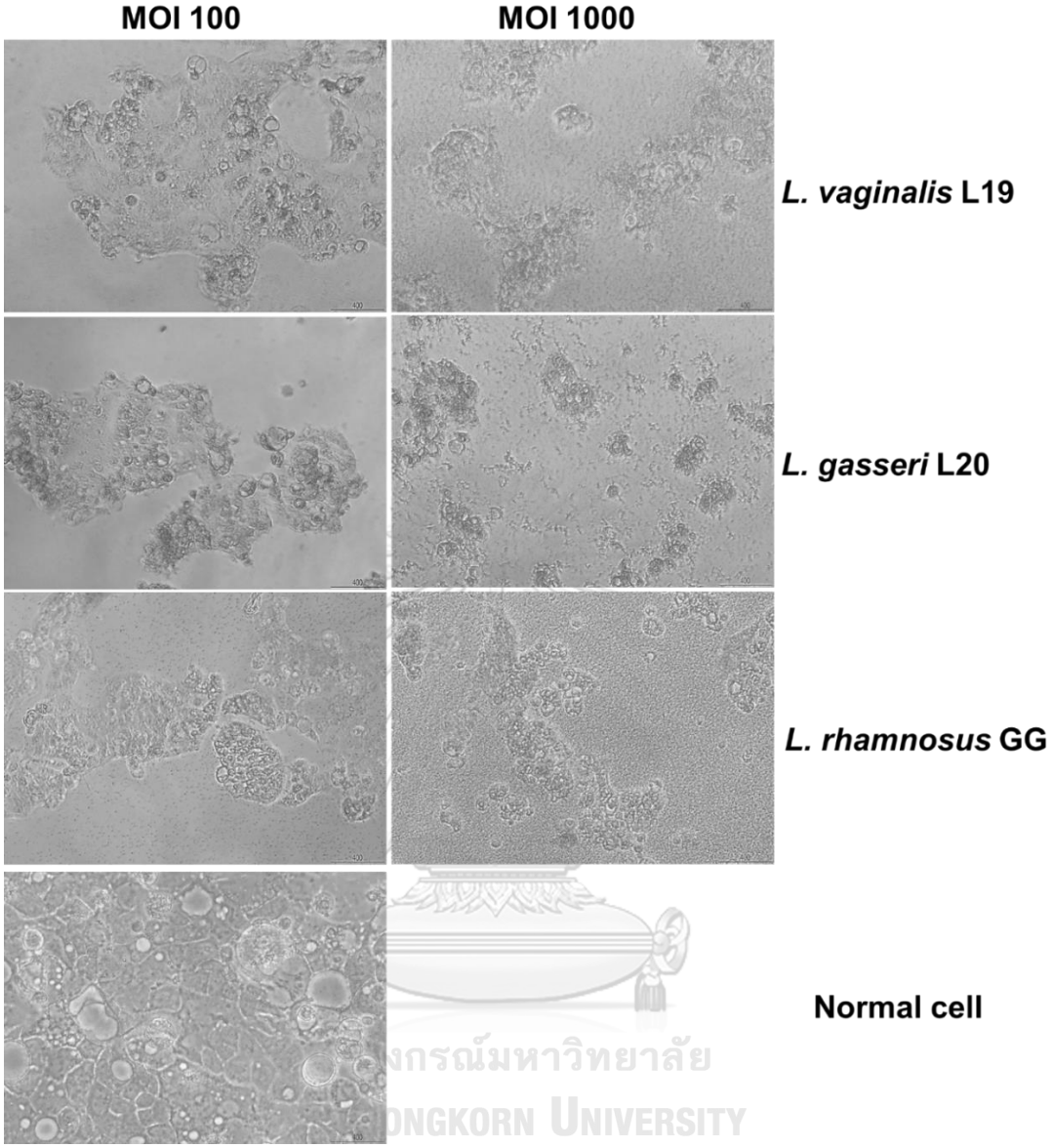


Figure 17 Morphology of Caco-2 cells after incubation with lactobacilli at the MOI of 100 and 1000 for 48 h. (Magnification × 100).

Normal cell is uninfected cell control.

At 24 h of incubation, *L. gasseri* L20 at an MOI of 100 significantly decreased cell viability of Caco-2 cells to 34.18 ± 6.12 % ($p < 0.001$), compared with the uninfected cell control (Figure 18). *L. rhamnosus* GG also significantly reduced cell viability to 51.58 ± 9.58 % ($p < 0.05$), and 29.84 ± 4.19 % ($p < 0.001$), at the MOI of 100 and 1000, respectively.

After 48 h of incubation, *L. gasseri* L20 significantly decreased cell viability of Caco-2 cells to 15.59 ± 5.04 % ($p < 0.0001$), and 8.64 ± 8.93 % ($p < 0.0001$), compared with the uninfected cell control at the MOI of 100 and 1000, respectively. Similarly, *L. rhamnosus* GG, at the MOI of 100 and 1000, significantly decreased cell viability to 42.63 ± 21.39% ($p < 0.01$), and 17.95 ± 13.58% ($p < 0.0001$), respectively.

In conclusion, *L. gasseri* L20 at both MOI significantly reduced cell viability of Caco-2 cells stronger than *L. vaginalis* L19. At the MOI of 100, *L. gasseri* L20 significantly reduced cell viability more than *L. vaginalis* L19 after 24 h ($p < 0.01$) and 48 h ($p < 0.01$) incubation. Furthermore, At the MOI of 1000, *L. gasseri* L20 also significantly decreased Caco-2 cell viability more than *L. vaginalis* L19 after 24 h ($p < 0.05$) and 48 h ($p < 0.001$) incubation. In addition, *L. rhamnosus* GG, at the MOI of 100, significantly reduced Caco-2 cell viability stronger than *L. vaginalis* L19 ($p < 0.05$) after 24 h incubation. At an MOI of 1000, *L. rhamnosus* GG showed significant reduction of cell viability at $p < 0.01$ after 24 and 48 h incubation.

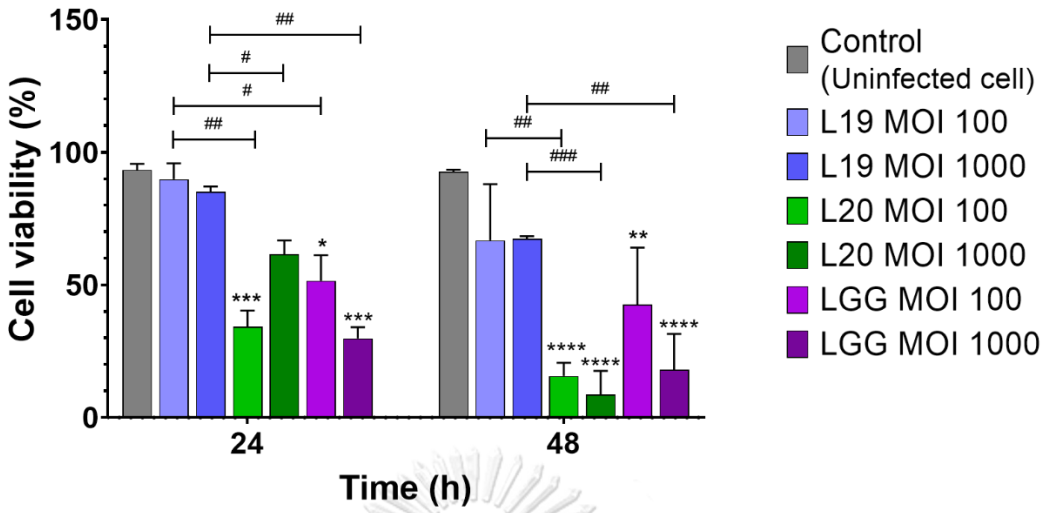


Figure 18 Anti-proliferative effect of viable lactobacilli at the MOI of 100 and 1000 on Caco-2 cells after 24 and 48 h incubation.

The results are from 2 independent experiments, each sample were performed in duplicate. Data are presented as mean ± SD (n=2). ***** represents the statistically significant difference between control and treated groups when $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). **#** represents the statistically significant difference between strains when $p < 0.05$ ($^{\#}$), $p < 0.01$ ($^{\#\#}$), $p < 0.001$ ($^{\#\#\#}$). L19: *L. vaginalis* L19; L20: *L. gasseri* L20; LGG; *L. rhamnosus* GG (ATCC 53103).

Part IV. Adhesion to intestinal epithelial cells

The ability of lactobacilli to adhere intestinal epithelial cells is considered as one of important characteristics affecting the beneficial activity of probiotics *in vivo*. This experiment used Caco-2 cell monolayer to study the adhesion of lactobacilli because the most prominent feature of polarized Caco-2 cells are microvilli covering their surface that form a typical intestinal brush border (Figure 18). The polarized Caco-2 cells in this study prepared by growing Caco-2 cells for 2 weeks and changed the medium every 2 days until differentiation.

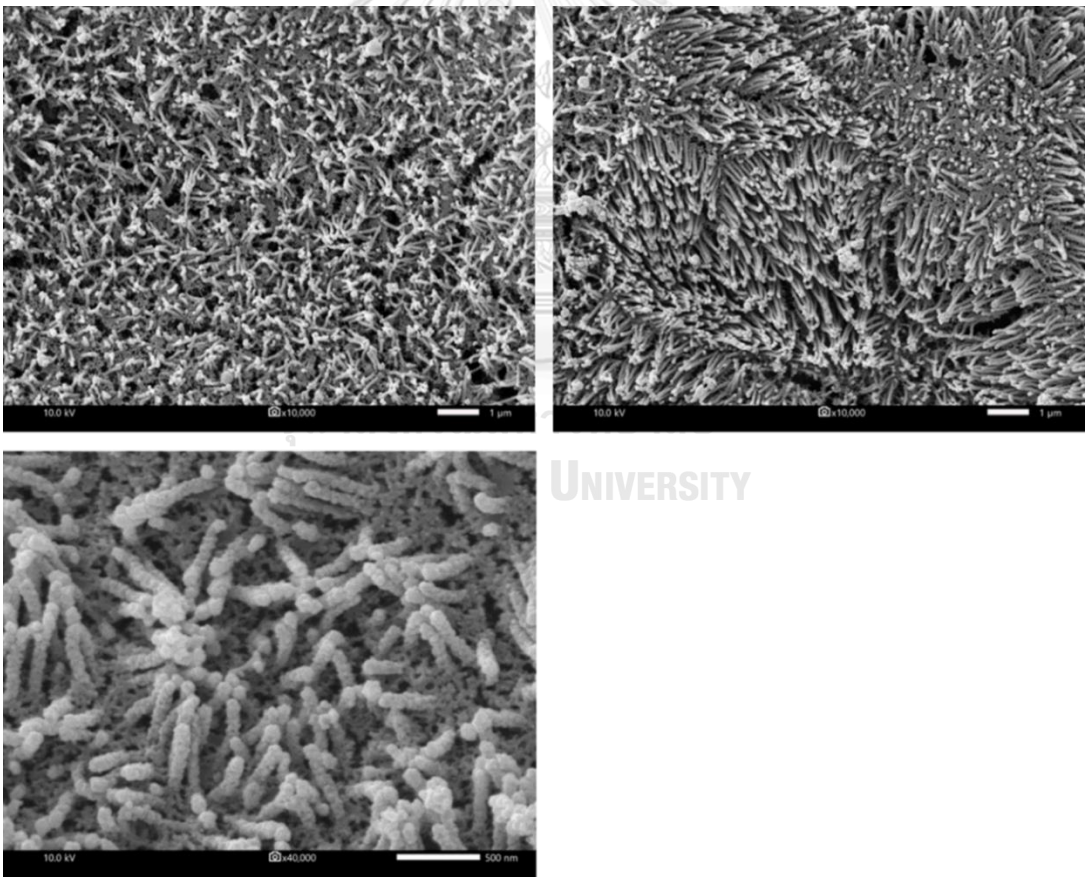


Figure 19 Scanning electron microscopy (SEM) images of the apical surface of differentiated Caco-2 cells after 14 days of culture.

The differentiated Caco-2 cells were incubated with lactobacilli at different MOI for 2 h. *L. rhamnosus* GG (ATCC 53103) strain was used as a positive control in this experiment because it was previously shown to strongly adhere to polarized Caco-2 cells (68).

L. vaginalis L19 and *L. gasseri* L20 demonstrated low adhesion ability to differented Caco-2 cells (Figure 20). *L. vaginalis* L19 at the MOI of 100 and 1000 showed 1.78 ± 0.99 and 1.88 ± 1.88 % adhesion, respectively. Likewise, *L. gasseri* L20 at the MOI of 100 and 1000 showed 1.12 ± 1.81 and 0.75 ± 0.76 % adhesion, respectively. As a positive control, *L. rhamnosus* GG was the most adhesive strain in this experiment. The adhesion percentage of *L. rhamnosus* GG at the MOI of 100 and 1000 was 4.75 ± 0.95 % and 7.04 ± 2.75 %, respectively. *L. vaginalis* L19 and *L. gasseri* L20 significantly adhered to differentiated Caco-2 cells lower than *L. rhamnosus* GG ($p < 0.01$).

In spite of mild adhesion to Caco-2 cells, the adhesion ability of *L. vaginalis* L19 and *L. gasseri* L20 to Caco-2 cells was detected by SEM (Figure 21).

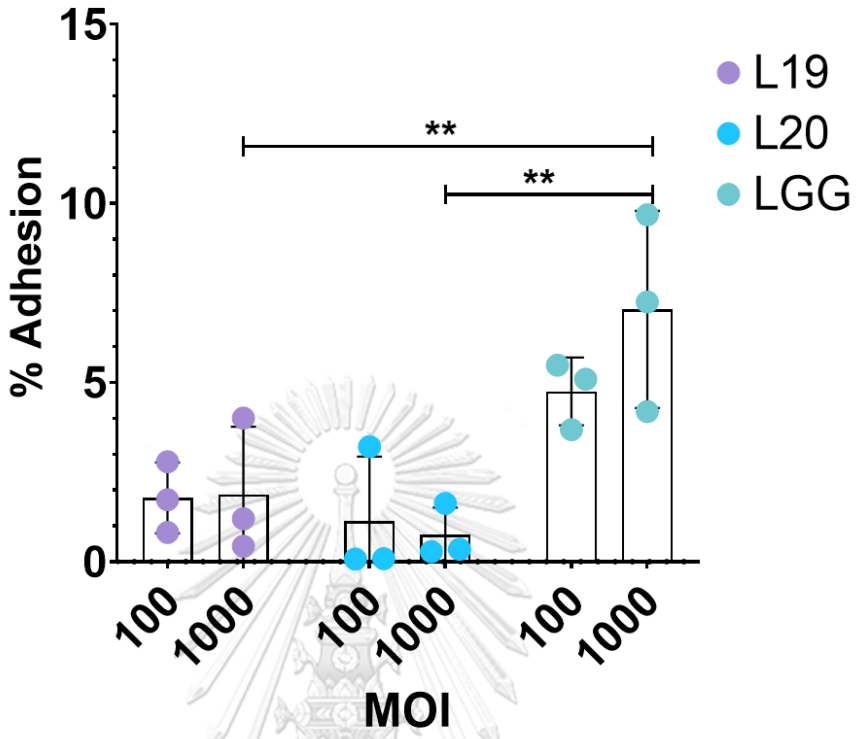


Figure 20 Adhesion of lactobacilli to differentiated Caco-2 cells at MOI of 100 and 1000. The differentiated Caco-2 cells were co-incubated with lactobacilli for 2 h. The result is obtained from 3 independent experiments and presented as mean ± SD (n=3). ***** represents the statistically significant difference between strains when $p < 0.01$ (**).

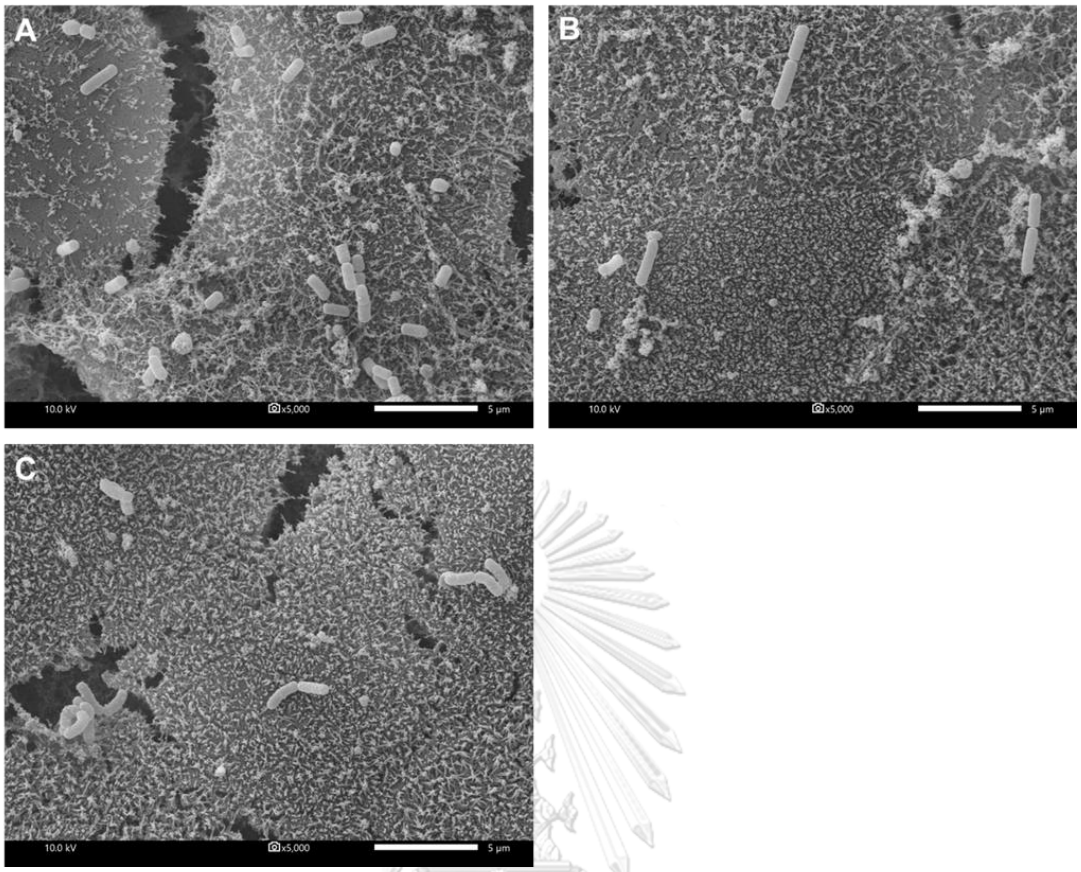


Figure 21 Scanning electron microscopy (SEM) images of the adherence of *L. vaginalis* L19 (A), *L. gasseri* L20 (B), and *L. rhamnosus* GG (C) to differentiated Caco-2 cells after 2 h co-incubation (magnification × 5,000).

CHAPTER VI

DISCUSSION

Colorectal cancer (CRC) is the third most common leading cause of cancer morbidity and mortality worldwide. It is associated with multifactorial risk factors. Current studies suggested that gut microbiota plays a role in CRC development (18). Numerous studies showed that probiotics can modulate homeostasis of gut microbiota (37). Therefore, the use of probiotics in the prevention of CRC is a promising approach. The effect of probiotics on CRC was investigated for a potential mechanism of CRC prevention (38). The major probiotics used belong to lactic acid bacteria (LAB), members of the genera *Lactobacillus* and *Bifidobacterium*. Probiotic treatment was shown to reduce risk factors for CRC through several possible mechanisms such as competition with pathogenic bacteria (69), inhibition of colorectal cancer cell proliferation *in vitro* (55, 70) and *in vivo* (71), promotion of apoptosis in cancer cells (72), and immune response modulation (40, 73).

This study aimed to identify lactobacilli with anticancer activity against CRC from thirty-nine human-derived *Lactobacillus* strains that were selected based on their immunomodulatory activity in previous studies (9, 10) (Table 1, Appendix B). Chronic inflammation is one of risk factors of CRC, although the mechanism leading to CRC is still unclear. Recent studies suggested that inflammatory cells and associated mediators such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), IL-

23, and reactive oxygen species involved CRC development by enhancing DNA damage of intestinal epithelial cells (74). Thus, anti-inflammation may be a mechanism for CRC prevention. Our 39 strains of lactobacilli previously showed anti-inflammatory effect, and showed ability to suppress TNF-α (Table 1, Appendix B). Therefore, anticancer activity other than anti-inflammation of these lactobacilli were examined in this study. The anti-proliferative effect on CRC cells was the first criterion used for screening anticancer activity of lactobacilli in this study. Firstly, *Lactobacillus* conditioned medium (LCM) were used to screen anti-proliferative activity on two CRC cell lines, HT-29 cells, and Caco-2 cells, which have been widely used to study the function of human intestinal cells *in vitro.* LCM was prepared (56) and used in this study either without pH adjustment at an approximate pH of 2-4 depending on the *Lactobacillus* strain or with pH adjustment to a pH of 7.6, which is the pH of DMEM (cell culture medium) (42) and the physiologic pH of human gut lumen (75). pH adjustment of LCM was used to determine whether acidic pH or secreted metabolites of LCM resulted in anti-proliferative effect on CRC cell lines.

To determine anti-proliferative effect of LCM on CRC cells, MTS assay was initially used for cytotoxicity detection because as a “one-step MTT assay”, it is more convenient and more rapid than MTT assay. Nevertheless, the final-step measurement of the absorbance showed highly variable results that was not correlated with the cell viability observed under a microscope. The absorbance detection of the MTS assay might be affected by the variation of original yellow-

brown color of LCM and variable pH of LCM from each strain. The MTT assay has an additional step of DMSO dissolution of formazan that help to eliminate the LCM color from the assay leading to more consistent and accurate results. Thus, MTT assay was alternatively used to determine the anti-proliferative effect of LCM in this study.

LCM from only 11 out of 39 strains of Lactobacillus seemed to have a certain degree of anti-proliferative activity against HT-29 cells, but only 2 out of 11 were significantly reduced (Figure 6 in appendix B). This result suggests that anti-proliferative effect of LCM on CRC cells is strain specific. Our finding is similar to a previous report showing different Lactobacillus species isolated from feces had different degrees of anti-proliferative effect on HT-29 cells (76). In our study, LCM obtained from L. vaginalis L19 and L. gasseri L20 significantly reduced the cell viability at the concentration of 2% LCM more than that from other strains. Either pH unadjusted or pH adjusted LCM from both strains was able to suppress the cell viability. Hence, not only acidic pH but also secreted metabolites of LCM from both strains have anti-proliferative potentials against HT-29 cells.

Next, LCM from *L. vaginalis* L19 and *L. gasseri* L20 was determined whether both LCM had anti-proliferative effect against more than one CRC cell types, so similar cytotoxicity study was performed using another cell type, Caco-2 cells. In contrast to HT-29 cells, Caco-2 cells were inhibited by pH adjusted but not pH unadjusted LCM from *L. vaginalis* L19 suggesting that secreted metabolites in LCM

contributed to anti-proliferative activity rather than its acidic pH. According to Chandel et al. (2019), the anti-proliferative effect of LCM from various *Lactobacillus* strains on HT-29 and Caco-2 cells was also different between cell types and probiotic strains used in the experiments (77). Therefore, LCM from *L. vaginalis* L19 and *L. gasseri* L20 might contain different secreted metabolites leading to distinct anti-proliferative effect on different CRC cell types. LCM used in this study exhibited only weak anti-proliferative effect, less than 10% cytotoxicity, on HT-29 and Caco-2 cells compared to some previous studies (67). The finding may be because only low concentrations of LCM (< 5% LCM) could be used in our experiments. Our preliminary results showed cytotoxic effect of MRS alone on the CRC cells. To exclude cytotoxic effect of MRS, the LCM concentration was selected for each CRC cell type based on the concentration that the MRS buffer did not inhibit cell viability.

Due to weak anti-proliferative activity of LCM on CRC cell lines, viable lactobacilli were further used to determine their cytotoxic effect on CRC cells. MTT assay was initially used to examine the anti-proliferative effect of viable lactobacilli against Caco-2 cells. After incubation with Caco-2 cells, lactobacilli were removed to exclude the interference of viable bacteria before measuring the absorbance in the MTT assay. Lactobacilli were not completely removed from each well despite several washing steps causing no correlation of the absorbance results with cell viability observed under a microscope. Therefore, flow cytometry was alternatively used to detect the viability of Caco-2 cells. This experiment used an uninfected cell

as a negative control and *L. rhamnosus LGG* (ATCC 53103), a commercial strain that were previously reported to inhibit proliferation of CRC cells, as a positive control (78). As expected, *L. rhamnosus* GG significantly decreased the cell viability at 24 and 48 h. Moreover, *L. vaginalis* L19 and *L. gasseri* L20 at an MOI of 1000 significantly decreased the cell viability of Caco-2 cells Furthermore, *L. gasseri* L20 also significantly decreased Caco-2 cell viability more than *L. vaginalis* L19 after 24 and 48 h incubation at the MOI of 100 and 1000. Similarly, previous studies demonstrated that viable lactobacilli had stronger anti-proliferative effect on the CRC cell viability than treated secreted metabolites in the conditioned media (42). Other parts of viable lactobacilli including cell-surface components may have the ability to inhibit CRC cell viability (79). However, this experiment performed in only two independent experiments, at least 3 independent experiments will be further performed to conclude significant anti-proliferative effect of our lactobacilli.

The ability to adhere to the intestinal epithelial cells was another potential property of probiotics to prevent CRC other than anti-proliferative effect was examined in this study. Adhesion is a key for colonization of microorganism to compete nutrients or adhesion with pathogenic microflora. Caco-2 cells are more similar to *in vivo* condition than HT-29 cells because Caco-2 cells can spontaneously differentiate to express morphological and functional characteristics of mature intestinal enterocytes (63), whereas HT-29 cells usually form multilayers of undifferentiated cells which do not commonly appear in the intestinal epithelia (80).

Therefore, this study used polarized and differentiated Caco-2 cells to determine the adhesive property of lactobacilli In the adhesion assay and scanning electron microscopy. Previous studies revealed the adhesive strains including *L. johnsonii* La1, *L. rhamnosus* GG (ATCC 53103), *L. casei* Shirota isolated from yakult and *L. casei* Imunitas had the ability to adhere differentiated Caco-2 cells (81). In addition, *L. rhamnosus GG,* the widely used commercial strain, have the ability to adhere to differentiated Caco-2 cells i*n vitro* (68) and also colonized the human gut that could promote faster recovery in patients with diarrhea (82). Therefore, *L. rhamnosus GG* was used as a positive control in this experiment.

L. vaginalis L19 and *L. gasseri* L20 showed a lower adhesion rate (< 5%) to differentiated Caco-2 cells than *L. rhamnosus* GG. *L. rhamnosus* GG showed 7.04 ± 2.75% adhesion to Caco-2 cells, which is similar to that shown in a previous report (83). In earlier studies, the ability to adhere to Caco-2 cells was shown to vary among probiotic strains and several probiotic strains also demonstrated weak adhesion to enterocyte-like Caco-2 cells (81, 84). Interestingly, Davoren, Liu et al (2019) found that 7 probiotic strains had various adhesive ability and were able to adhere to the goblet cell-like LS174T cells, modified epithelial cells that secrete mucus on the surface, better than to enterocyte-like Caco-2 cells (85). These data suggest that *Lactobacillus* strains were able to adhere to different intestinal epithelial cell types. The *in vitro* Caco-2 cell model is the form of intestinal epithelium that lacks the mucus layer and unstrirred water layer (86). In addition, the intestinal epithelium *in*

vivo not only contains enterocytes, but also includes goblet cells, that produce and secrete mucins to cover the gastrointestinal tract, which are different from *in vitro* cell lines.

The limitation of our study was to evaluate only certain proterties of lactobacilli required for anticancer activity. Our experiments focused on the ability to inhibit cell proliferation and to adhere CRC cells. However, probiotics used for CRC prevention should have more properties than those determined in our study, for example, the ability of binding and degradation of carcinogenic compounds presented in the intestinal lumen, change in the metabolic activity of the intestinal microbiota, and production of compounds with anticarcinogenic activity such as short-chain fatty acids (SCFAs) and conjugated linoleic acid (CLA) (39). Moreover, previous studies found the effect of pH on cell death mediated by SCFA (87). The study examined the effect of *Propionibacterium freudenreichii* subsp. *freudenreichii* strain TL142 on HT-29 cells by comparing the supernatants from alive and heat-killed propionibacteria with those adding SCFAs (acetate and propionate), SCFAs alone adjusted pH to 7.5, and media adjusted pH to 7.5. The result showed killed propionibacteria with SCFAs did not reduce cell viability. The more acidic pH of SCFAs showed higher reduction of cell viability. Therefore, acidic pH potentiated the anti-proliferative effect of SCFAs. However, the secreted metabolites in our findings were unknown because we have not identified the metabolites in each LCM. Thus, we cannot conclude that the anti-proliferative effect against HT-29 cells that

occurred apart from acidic pH is the result of SCFAs. Further study should focus on the effect of secreted metabolites and SCFAs produced from our *Lactobacillus* strains on CRC cell viability. Furthermore, individual strain of *Lactobacillus* was evaluated in our study. A previous study demonstrated that a combination of *Lactobacillus* strains could synergistically inhibit tumor growth in a CRC mouse model better than individual probiotic strain (54). Therefore, the combination of our *Lactobacillus* strains should be further evaluated to determine whether enhanced anti-proliferative effect on CRC cells can be achieved. In the anti-proliferative study, the mechanism of cell death is unknown. We need to further investigate to distinguish whether the cell death process induced by our lactobacilli is apoptosis or necrosis. Apoptosis is programmed cell death to eliminate cell with no subsequent inflammation, therefore the induction of apoptosis is a potential mechanism of probiotics for CRC prevention. Regarding the adhesion assay, only one cell type, i.e. enterocyte-like Caco-2 cells, was used. Therefore, more than one intestinal epithelial cell types, including goblet cells, or combination of more than one cell types should be used to evaluate the adhesion ability of probiotic strains. However, in case of low adhesion ability, regular intake and high dose of these probiotic strains may be required to maintain gut symbiosis and confer anticancer activity on the intestinal tract.

In conclusion, our study found 2 out of 39 Thai population-origin and anti-inflammatory strains of *Lactobacillus* spp., *L. vaginalis* L19 and *L. gasseri* L20, that

showed anti-proliferative effect on two CRC cell types, HT-29 and Caco-2 cells. Intact and viable lactobacilli demonstrated much stronger anti-proliferative effect than their corresponding LCM derived from these two strains. In addition, *L. vaginalis* L19 and *L. gasseri* L20 showed the ability to adhere enterocyte-like Caco-2 cells. These two lactobacilli might be useful for prevention of colorectal cancer especially in Thai patients in the future.

APPENDIX A

MATERIALS

BUFFER AND REAGENT

1. deMan Rogosa Sharpe (MRS) agar

Typical formula

Peptone mixture	18.0 g/L
Yeast extract	4.0 g/L
Glucose	20.0 g/L
Tween 80	1.0 g/L
Di-potassium hydrogen phosphate	2.0 g/L
Tri-ammonium citrate	2.0 g/L
Sodium acetate anhydrous	3.0 g/L
Magnesium sulphate 7 H_20	0.2 g/L
Manganese sulphate anhydrous	0.034 g/L
Agar	12.0 g/L
Distilled water	1,000 ml

pH approximately 6.2 ± 0.2

2. MRS broth

MRS medium	52 g/L
Distilled water	1,000 ml

3. 20% MRS glycerol stock solution

MRS medium	52 g
Glycerol	200 ml
Distilled water	1,000 ml

4. 0.85% Normal saline solution

Sodium chloride (NaCl)	8.5 g/L
Distilled water	1,000 ml

5. Cell culture medium for HT-29 cell line (100 ml)

Dulbecco's Modified Eagle's Medium (DMEM)	89 ml
10% Fetal Bovine Serum (FBS)	10 ml
1% (w/v) penicillin and streptomycin	1 ml

6. Cell culture medium for Caco-2 cell line (100 ml)

DMEM	78 ml
20% FBS	20 ml
1% HEPES	1 ml
1%Sodium pyruvate	1 ml

7. Flow Cytometry Staining Buffer (100 ml)

FACS	50 ml
FBS	1 ml
PBS	49 ml

APPENDIX B

Table 1 Thirty-nine *Lactobacillus* strains that their *Lactobacillus* conditioned medium (LCM) has the ability to suppress TNF-alpha production in *E. coli* LPS-induced THP-1 cells [unpublished data and (9, 10)].

No.	*Lactobacillus* species	Strains	Origins	%TNF inhibition	Reference
1	*L. gasseri*	L2	Infant feces	27.93	unpublished
2	*L. gasseri*	L3	Infant feces	33.54	unpublished
3	*L. salivarius*	L8	Infant feces	26.77	unpublished
4	*L. gasseri*	L10	Infant feces	65.27	unpublished
5	*L. salivarius*	L11	Infant feces	48.92	unpublished
6	*L. salivarius*	L17	Infant feces	24.55	unpublished
7	*L. vaginalis*	L19	Infant feces	61.13	unpublished
8	*L. gasseri*	L20	Infant feces	51.32	unpublished
9	*L. salivarius*	L22	Infant feces	28.01	unpublished
10	*L. salivarius*	L23	Infant feces	41.14	unpublished
11	*L. gasseri*	L26	Infant feces	23.56	unpublished
12	*L. gasseri*	L29	Infant feces	28.54	unpublished
13	*L. gasseri*	L30	Infant feces	23.42	unpublished
14	*L. gasseri*	L32	Infant feces	36.53	(9)
15	*L.rhamnosus*	L33	Infant feces	25.66	(9)
16	*L. rhamnosus*	L35	Infant feces	53.19	(9)
17	*L. casei group*	B13	Human gastric biopsy	28.26 *	(10)
18	*L. gasseri*	XB68	Human gastric biopsy	52.2 *	(10)
19	*L. plantarum*	B90	Human gastric biopsy	47.32 *	(10)
20	*L. gasseri*	XB94	Human gastric biopsy	24.86 *	(10)
21	*L. salivarius*	B101	Human gastric biopsy	38.69 *	(10)
22	*L. casei group*	B106	Human gastric biopsy	51.93 *	(10)
23	*L. casei group*	B107	Human gastric biopsy	11.49 *	(10)
24	*L. salivarius*	B109	Human gastric biopsy	16.35 *	(10)
25	*L. plantarum*	B6	Human gastric biopsy	25.58*	(10)
26	*L. plantarum*	B7	Human gastric biopsy	35.41*	(10)
27	*L. salivarius*	B8	Human gastric biopsy	31.19*	(10)

28	*L.salivarius*	B21	Human gastric biopsy	14.98*	(10)
29	*L. gasseri*	XB41	Human gastric biopsy	13.19*	(10)
30	*L. salivarius*	B47	Human gastric biopsy	10.04*	(10)
31	*L. gasseri*	XB48	Human gastric biopsy	16.62*	(10)
32	*L. gasseri*	XB58	Human gastric biopsy	40.61*	(10)
33	*L. plantarum*	B67	Human gastric biopsy	30.08*	(10)
34	*L. plantarum*	B70	Human gastric biopsy	47.70*	(10)
35	*L.salivarius*	B73	Human gastric biopsy	22.10*	(10)
36	*L. salivarius*	B74	Human gastric biopsy	29.03*	(10)
37	*L. gasseri*	XB95	Human gastric biopsy	10.29*	(10)
38	*L. gasseri*	XB96	Human gastric biopsy	8.37*	(10)
39	*L. salivarius*	B52	Human gastric biopsy	23.98*	(10)

* indicate statistically significant suppression

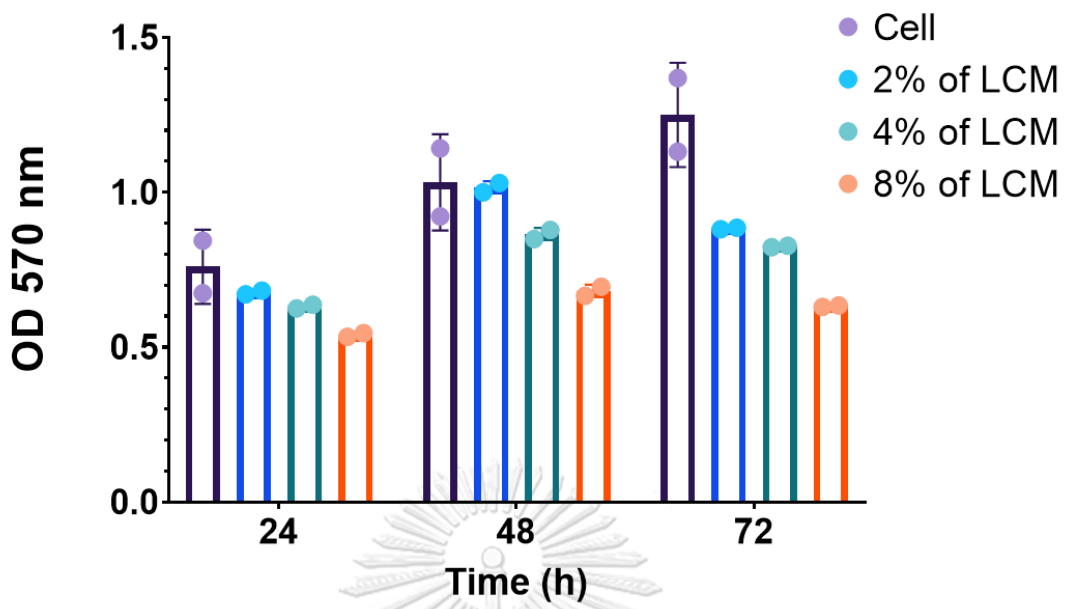


Figure 1 The effect of different concentrations of MRS medium on cell viability of Caco-2 cell lines at 24, 48 and 72 h. MTT assay was performed to detect cell viability and the absorbance value was measured at a wavelength of 570 nm (OD 570). All data are from 3 independent experiments. Data are presented as mean ± SD *(n=3)*.

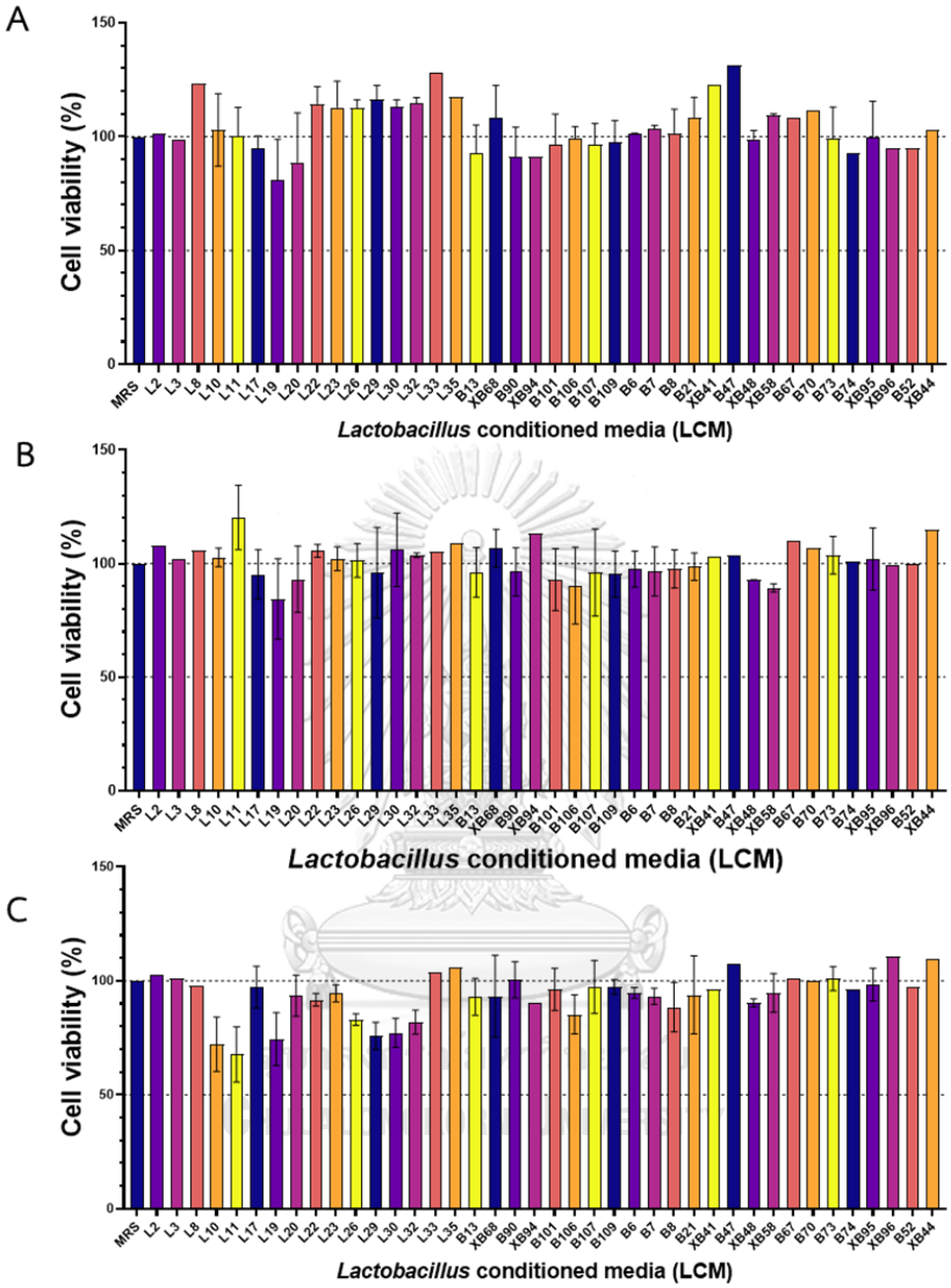


Figure 2 Anti-proliferative effect of thirty-nine pH unadjusted *Lactobacillus* conditioned media (LCM, pH 2-4) at the concentration of 1% on the viability of HT-29 cells at 24 (A), 48 (B), and 72 h (C) incubation.

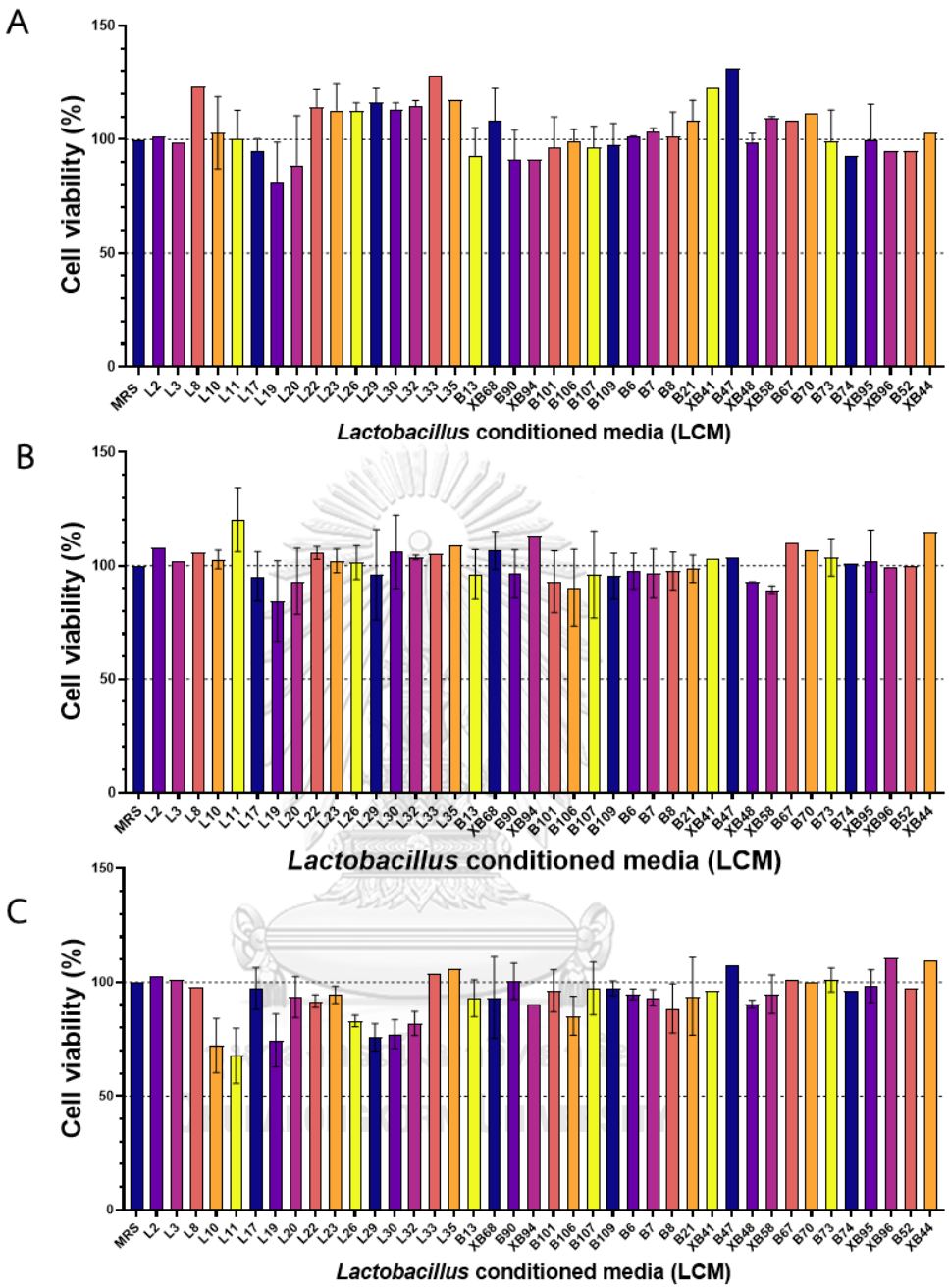


Figure 3 Anti-proliferative effect of thirty-nine pH adjusted *Lactobacillus* conditioned media (LCM, pH adjusted to 7.6) at the concentration of 1% LCM on the viability of HT-29 cells at 24 (A), 48 (B), and 72 h (C) incubation.

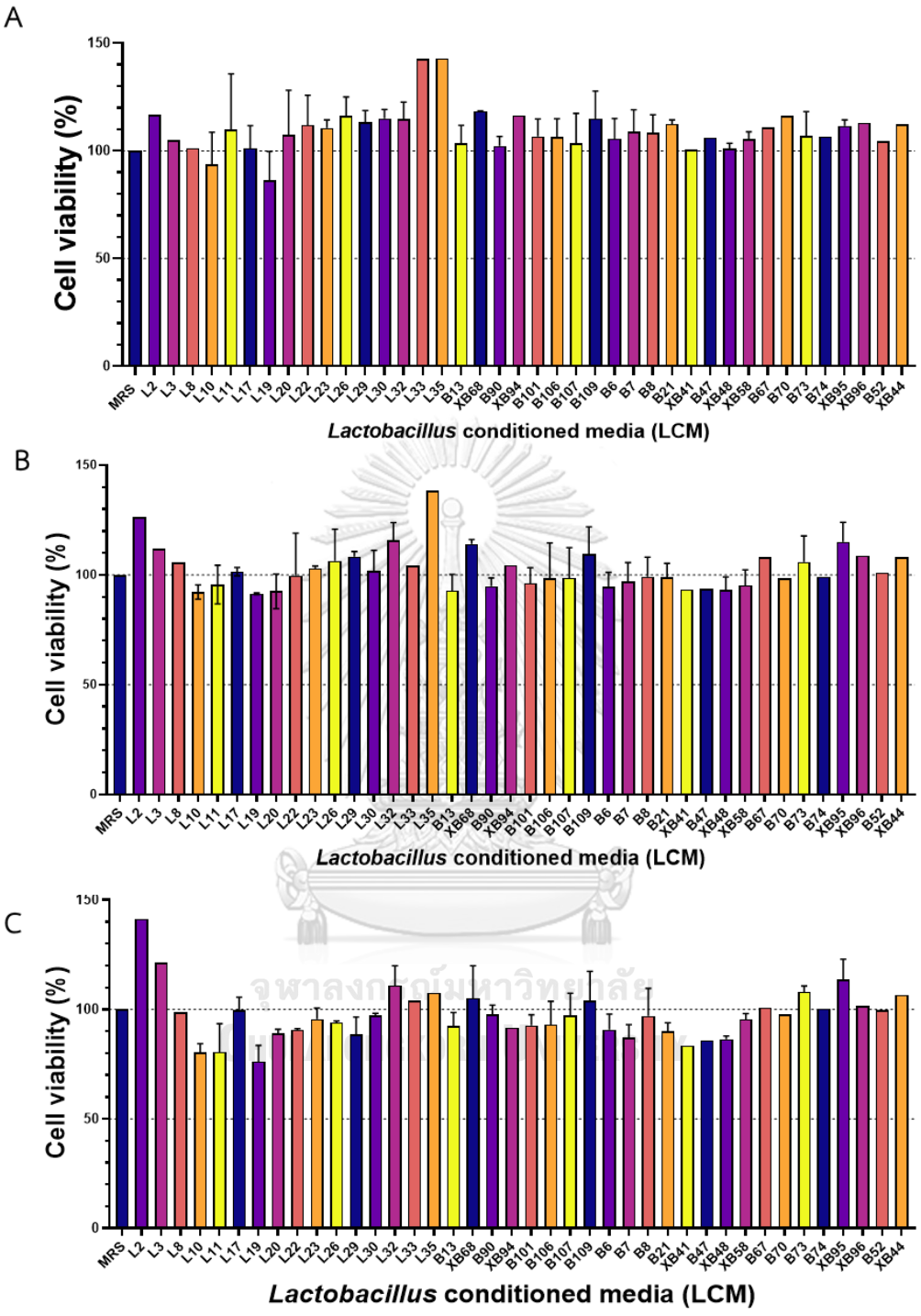


Figure 4 Anti-proliferative effect of thirty-nine pH unadjusted *Lactobacillus* conditioned media (LCM, pH 2-4) at the concentration of 2% on the viability of HT-29 cells at 24 (A), 48 (B), and 72 h (C) incubation.

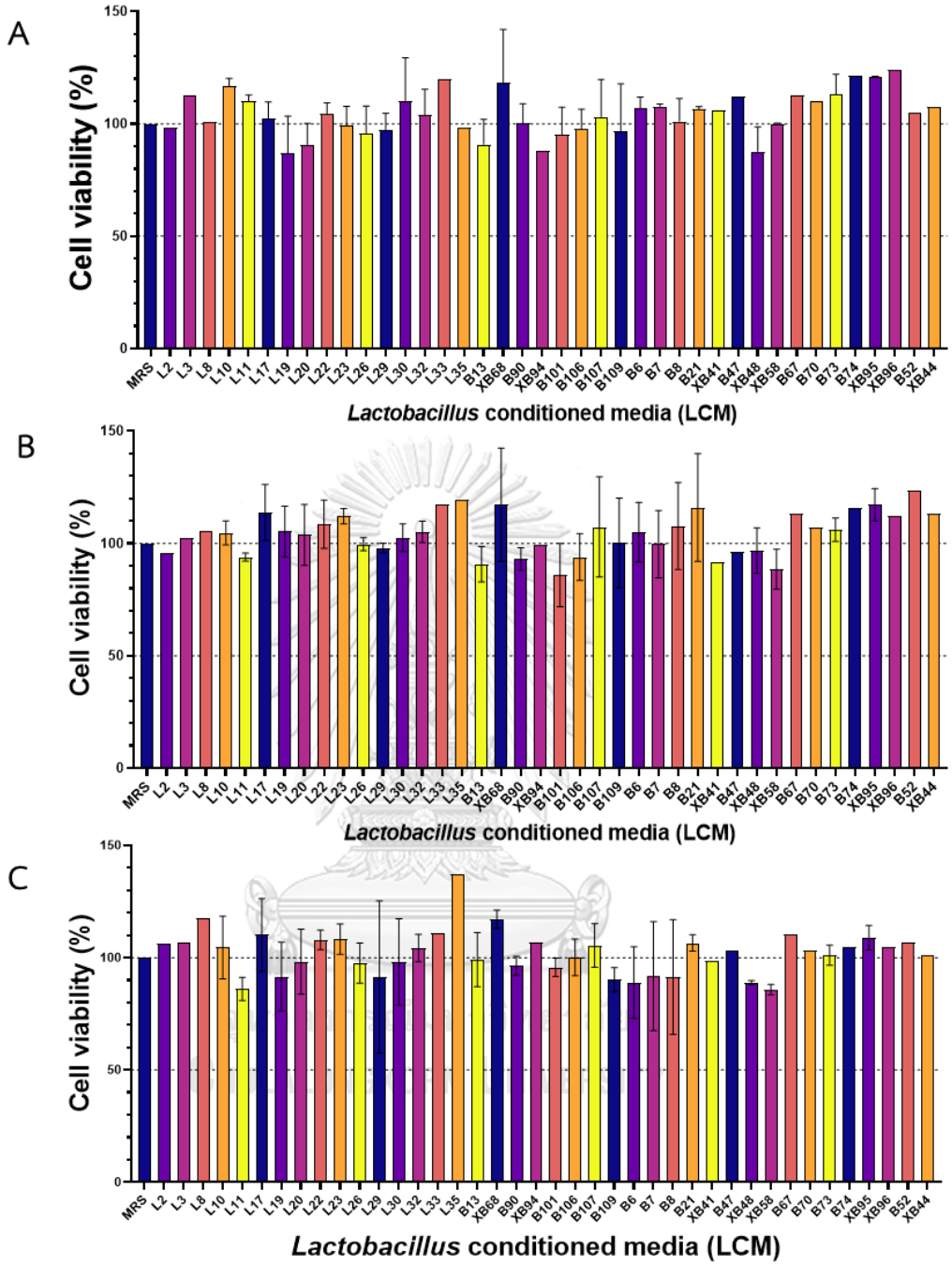


Figure 5 Anti-proliferative effect of thirty-nine pH adjusted *Lactobacillus* conditioned media (LCM, pH adjusted to 7.6) at the concentration of 2% on the viability of HT-29 cells at 24 (A), 48 (B), and 72 h (C) incubation.

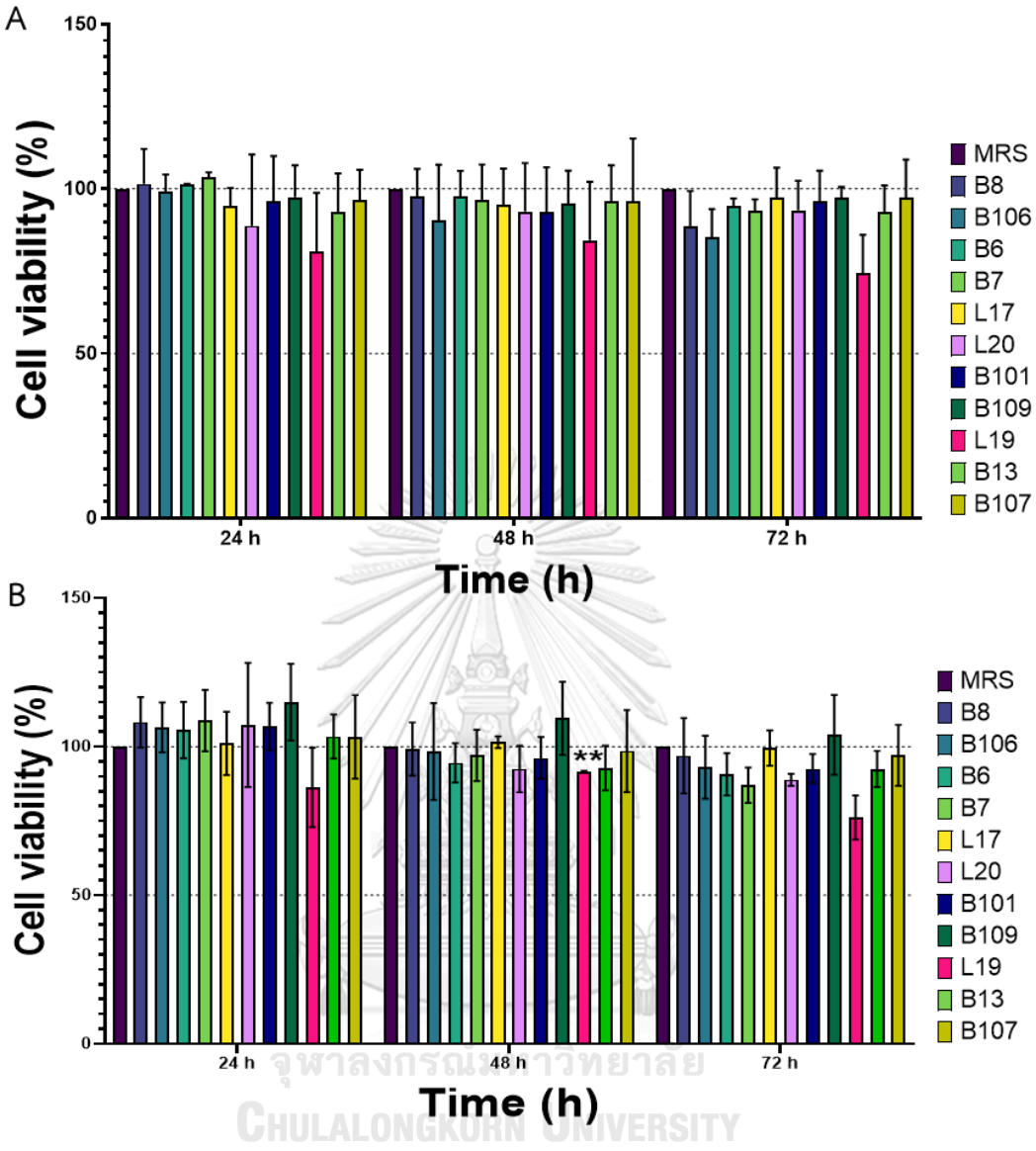


Figure 6 Anti-proliferative effect of eleven pH unadjusted *Lactobacillus* conditioned media (LCM, pH 2-4) at the concentration of 1% (A) and 2% (B) on the viability of HT-29 cells at 24 (A), 48 (B), and 72 h (C) incubation.

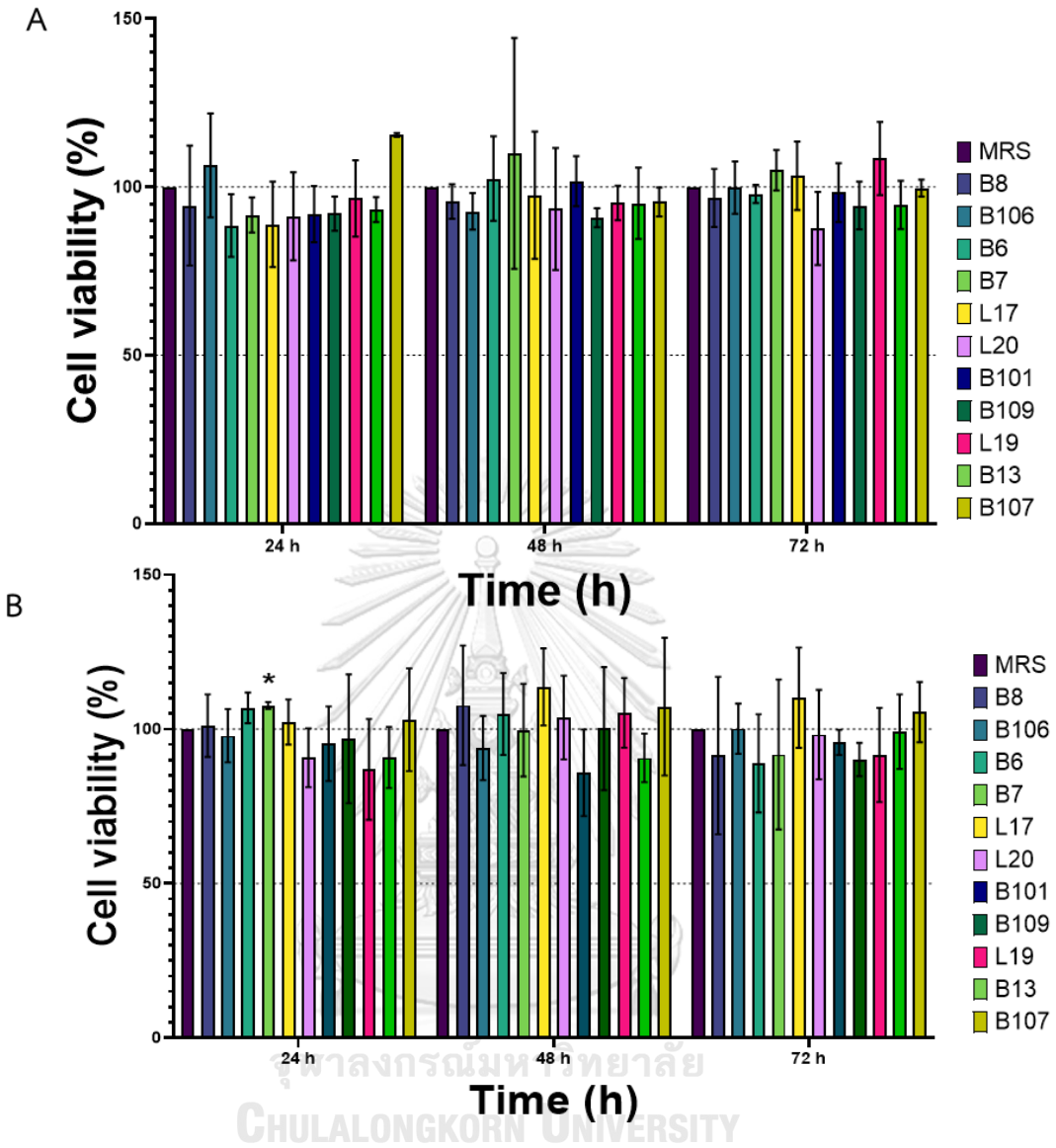


Figure 7 Anti-proliferative effect of eleven pH adjusted *Lactobacillus* conditioned media (LCM, pH adjusted to 7.6) at the concentration of 1% (A) and 2% (B) on the viability of HT-29 cells at 24, 48, and 72 h incubation.

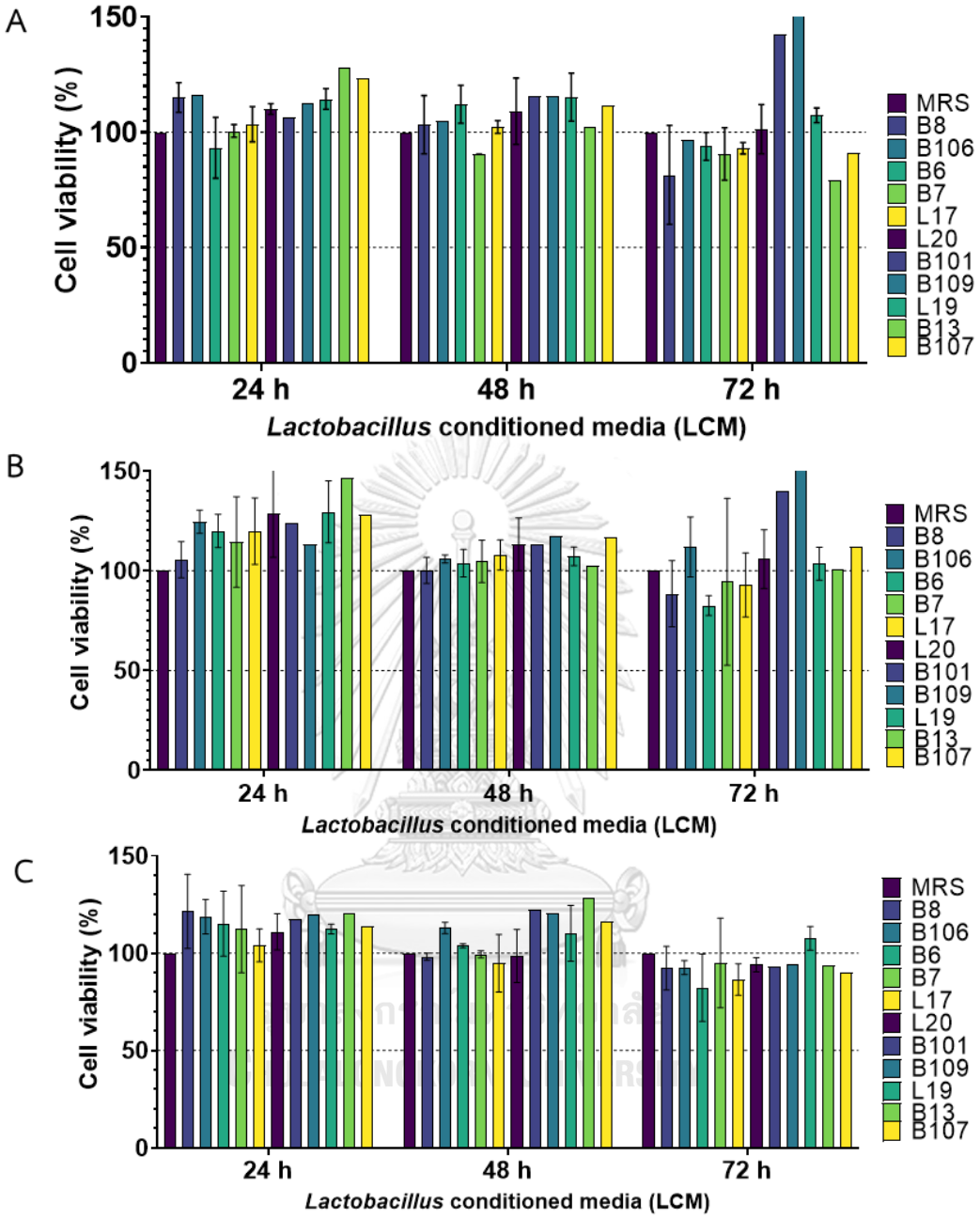


Figure 8 Anti-proliferative effect of eleven pH unadjusted Lactobacillus conditioned media (LCM, pH 2-4) at the concentration of 1% (A), 2% (B), and 4% (C) on the viability of Caco-2 cells at 24, 48, and 72 h.

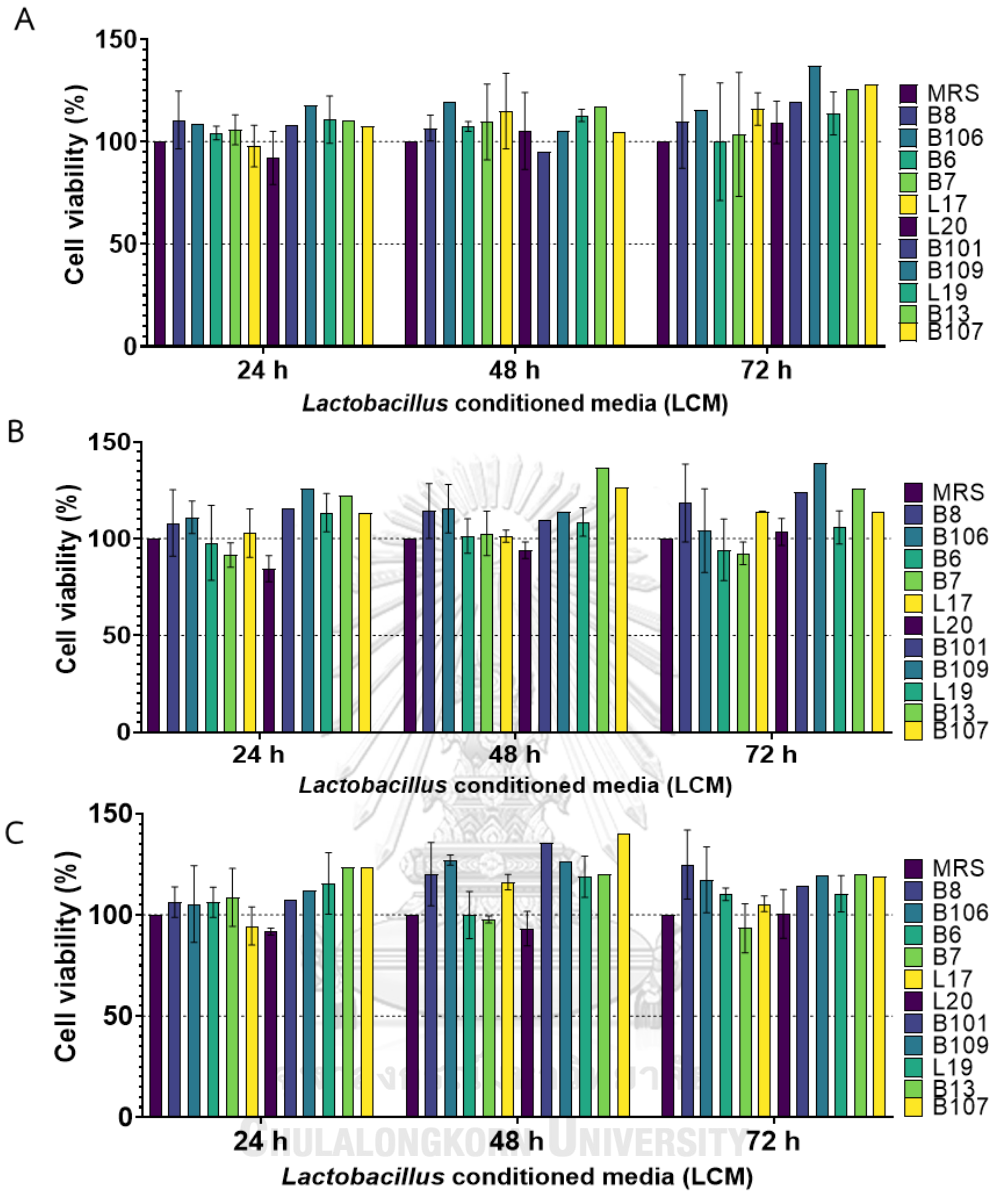


Figure 9 Anti-proliferative effect of eleven pH adjusted *Lactobacillus* conditioned media (LCM, pH adjusted to 7.6) at the concentration of 1% (A), 2% (B), and 4% (C) on the viability of Caco-2 cells at 24, 48, and 72 h incubation.

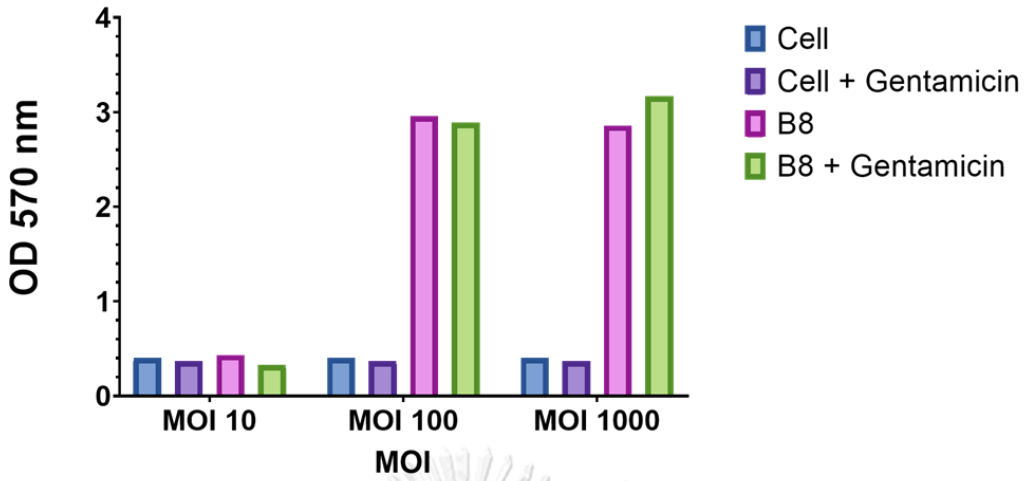


Figure 10 MTT assay was performed to detect cell viability of Caco-2 cells when co-infected with Lactobacillus strain at the MOI of 10, 100, and 1000 in the presence or absence of gentamicin treatment. The absorbance value was measured at a wavelength of 570 nm (OD 570). B8; *L. salivarius* B8.

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