National Journal of Physiology, Pharmacy and Pharmacology

RESEARCH ARTICLE

Screening of lactic acid bacteria with immune modulating property, and the production of lactic acid bacteria mediated fermented soymilk

Sasithorn Sirilun[1], Chaiyavat Chaiyasut[1], Periyanaina Kesika[1], Sartjin Peerajan[2], Bhagavathi Sundaram Sivamaruthi[1]

[1]Faculty of Pharmacy, Innovation Center for Holistic Health, Nutraceuticals, and Cosmeceuticals, Chiang Mai University, Chiang Mai, Thailand, [2]Health Innovation Institute, Chiang Mai, Thailand

Correspondence to: Bhagavathi Sundaram Sivamaruthi, E-mail: sivasgene@gmail.com

Received: September 01, 2017; **Accepted:** September 26, 2017

ABSTRACT

Background: Lactic acid bacteria (LAB) are reported to have immune activating property. The genetically modified LAB strains producing interleukin (IL) -10 were under consideration for the treatment of inflammatory bowel diseases (IBD). Soybean is rich in necessary nutrients, and the fermented soybean products are known for its health benefits. **Aims and Objectives:** The current study was aimed to screen LAB with the immune activating property, and production and evaluation of LAB mediated fermented soymilk product. **Materials and Methods:** Screening of LAB from the fermented foods collected in Chiang Mai. To select a potent LAB strain, the LAB isolates were scrutinized for acid tolerant, bile tolerant, growth rate, antimicrobial ability, adhesion property, and induction of IL-10 production. The physical properties of fermented soymilk (FSM) were evaluated, and *in vitro* activation of IL-10 by FSM was assessed using Caco-2 cell line. **Results:** About 742 LAB strains were isolated from 335 fermented food samples. Based on the tested properties, LAB33 was selected as a potent LAB strain, and used for the fermentation of soymilk for 30 days. The LAB33 FSM was superior in quality regarding probiotic nature and microbiological safety. **Conclusion:** The results of the study suggested that LAB33 is the potent probiotic strain for the production of soymilk-based fermented functional food supplements for the betterment of IBD patients. Further, strain identification is required to register the strain in a public repository, and sustainable utilization for the benefit of mankind.

KEY WORDS: Fermented Soymilk; Immune Activation; Interleukin-6; Interleukin-10; Lactic Acid Bacteria

INTRODUCTION

Lactic acid bacteria (LAB) are Gram-positive, non-spore forming, cocci, or bacilli bacteria. LAB are the common name for genera such as *Lactobacillus*, *Pediococcus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Enterococcus*, *Carnobacterium*, *Oenococcus*, *Vagococcus*, *Tetragenococcus*, and *Weissella*, even though many bacterial genera produce lactic acid during fermentation.

Access this article online

Website: www.njppp.com

DOI: 10.5455/njppp.2017.7.0933926092017

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LAB have a defect in biosynthetic ability. Thus, complex enriched media is required for the culturing of LAB. Most of the LAB strains are free-living or advantageous associated with animals, and rarely LAB becomes opportunistic pathogens. LAB are commonly found in dairy products and putrefying plant materials. LAB are normal flora of humans, primarily harbored in the intestinal tract, oral cavity, and the vaginal region.

LAB are known for the maintenance and enrichment of normal flora of the system, anti-diabetic,

antihyperlipidemic, anti-colitic, anti-carcinogenic activity.[1-6]

LAB have a positive stimulus on ingestion and the immune system.[7] LAB are reported for non-specific activation of the host's defense system.[1] *Lactococcus lactis* is used for cytokine delivery. The genetically engineered *L. lactis*, in which the thymidylate synthase gene has been replaced by human interleukin (IL) -10 gene, the strain is growth restricted and able to produce IL-10 in the human system. The administration of genetically modified *L. lactis* nullified the medical discomfort in the Crohn's disease patients.[8-10]

Soybean and its associated products offer proteins, without cholesterol and lactose, which are favorable for lactose intolerance patients and vegetarians. Akhuni, cheonggukjang, doenjang, doubanjiang, douchi, fermented bean paste, gochujang, jajangmyeon, mianchi, miso, natto, tofu, pickled tofu, soy sauce, stinky tofu, tamari, tauchu, tauco, tempeh, tianmianjiang, and tuong are some of the soybean-based fermented and non-fermented foods commonly used in Asian countries such as China, Japan, Korea, India, Indonesia, and Vietnam.

The health benefits of the soybean and its compounds have been described previously. The fermented soy products, particularly fermented by probiotic LAB, have been reported for anti-obesity properties, controlling and suppressing the serum cholesterol level, inhibiting the pathogen multiplication, modifying the immune system, and diminishing the risk of atherosclerosis and cardiovascular diseases.[11-15]

As described earlier, engineered LAB strains were used to enhance the immune system against disorders and diseases, chiefly for gastrointestinal diseases. Soymilk is the key ingredient for making several fermented soy products. Thus, the current study was designed and executed the screening of LAB from fermented foods of Thailand with the immune activating property, and the production of fermented soymilk (FSM) using isolated LAB strain.

MATERIALS AND METHODS

Sample Collection and Isolation of LAB

The samples of fermented plant foods (like fermented papaya broth, fermented emblica broth, fermented banana broth, fermented noni broth, fermented houttuynia broth, fermented black galingale broth, fermented citronella grass broth, fermented kaffir lime broth, fermented wild fruits, fermented pickled vegetable, fermented beans, rotten beans, tea leaves, pickled garlic, pickled turnip greens, pickled lemon, pickled peach, pickled cleome, pickled onion, pickled cabbage, pickled radish, and pickled bamboo) were collected at local market of Chiang Mai, Thailand. Then, LAB were isolated from the collected samples as described previously.[16]

Acid Tolerant and Bile Salt Resistance Test

The isolated LAB strains were inoculated in MRS (de Man, Rogosa, and Sharpe) broth with pH 3 and incubated at 35°C for 24 h. After incubation, the medium was examined for the growth of LAB strain by spectrophotometric analysis.

Likewise, to test the bile salt tolerance, LAB isolates were inoculated in MRS broth with 0.15% and 0.30% of bile salt and incubated at 35°C for 24 h. After incubation, the medium was examined for the growth of LAB strain by spectrophotometric analysis.

Antimicrobial Activity of LAB Isolates and FSM

The antimicrobial activity of isolated LAB strains and FSM were assessed against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus*, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella* Typhi, *Shigella sonnei*, and *Candida albicans* ATCC 90028 by agar well-diffusion method.[17] Briefly, tryptic soy broth for bacteria and sabouraud dextrose broth for yeast were prepared, and the culture was inoculated by spread plate method. Then, agar wells were created on the medium using well puncher. About 100 µL of the cell-free supernatant of LAB culture was poured in the agar well and incubated at 35°C for 24 h. After incubation, the zone of clearance was measured.

Assessment of Adherence Property of LAB and Induction of Cytokine Production

Caco-2 cells (1×10^4 cell/mL), and LAB strains (1×10^8 CFU/mL) were used for the assessment of adherence property of selected LAB strains and for the evaluation of the cytokine-inducing ability of LAB as detailed in the previous studies.[18,19] The production of cytokines (IL-6, pro-inflammatory cytokines; IL-10, anti-inflammatory cytokines) was determined by enzyme-linked immunosorbent assay (ELISA) using Quantikine ELISA kit (R&D Systems, USA). The FSM samples, which contain LAB strain, were also detected for the induction of the cytokine production in Caco-2 cells.

Generation Time and Specific Growth Rate of LAB

The multiplication time and specific growth rate of LAB were calculated as detailed previously.[17]

Physical and Biochemical Analysis of Selected LAB Strains

The selected LAB strains were assessed for the shape, cell wall composition (Gram staining), catalase production, carbohydrate, and sugar fermentation (amygdalin, arabinose, cellobiose, esculin, fructose, galactose, glucose, lactose, maltose, mannitol, raffinose, rhamnose, ribose, sorbitol,

sucrose, and trehalose) as described in the Bergey's Manual of Determinative Bacteriology.[20]

Fermentation of Soybean Milk

Soymilk was extracted from fresh soybean by soaking the beans for overnight in sterile water (1:10) and crushed the soaked beans. The soybean extract was filtered (sterile muslin cloth) and pasteurized at 69 ± 3°C for 30 min. Then, 1% of LAB starter culture (~10^7 CFU) was introduced to the sterile soymilk and incubated at room temperature in an aseptic condition for 30 days. The soymilk without starter culture was used as control.

Quantification of Lactic Acid and pH in FSM

The quantity of the lactic acid during the fermentation process was measured by (High-Performance Liquid Chromatography; Advanced Chromatography Technologies, Scotland),[21] and the pH value of the sample was determined by pH meter (Metrohm 691).[21]

Microbiological Analysis of FSM

The microbial safety of the FSM was assessed by measuring the total microbial count, yeast and mold, MPN coliforms, *E. coli*, *S. aureus*, *Clostridium perfringens*, *P. aeruginosa,* and *Salmonella*. by a plating method. Specific media was used for the growth of the respective microbes. Trypticase soy agar and sabouraud dextrose agar media (HiMedia) were used for culturing bacteria, and yeast and mold, respectively.[22]

Statistical Analysis

All the experiments were performed in triplicates, and the values were denoted as a mean ± standard deviation. Data were analyzed using SPSS 17.0 for Windows® (2009 SPSS Inc., Chicago, IL, USA) by analysis of one-way analysis of variance. Differences were measured as significant at $P < 0.05$.

RESULTS

The fermented food samples (335 in total) were collected from several parts of Thailand. All the samples were processed, and about 742 LAB strains were obtained. The LAB strains were categorized based on the shape by microscopic observations. About 183 and 559 isolates were found as cocci, and bacilli, respectively (Table 1).

LAB strains were evaluated for acid and bile salt tolerance. The results showed that about 36.79, and 63.21 % of LAB isolates are acid tolerant, and non-acid tolerant, respectively (Figure 1a). About 97.44 and 67.40% of isolates are tolerant to 0.15, and 0.15-0.30% of bile salt, respectively. About 2.56% of isolates were found to be non-bile salt tolerant (Figure 1b).

Table 1: Sample collection area, number of samples collected, number of isolates LAB strains, and the shape of the bacterium

Place of sample collection	Number of samples	Number of LAB isolates	Shape of the bacterium	
			Cocci	Bacilli
Chiang Mai	25	109	26	83
Chiang Rai	10	72	18	54
Payao	10	17	-	17
Mae Hong Son	120	183	81	102
Lamphun	5	-	-	-
Lampang	5	-	-	-
Tak	10	24	-	24
Phetchabun	10	37	-	37
Northeastern provinces	100	242	58	184
Central and eastern provinces	30	22	-	22
West and south provinces	10	36	-	36
Total	335	742	183	559

LAB: Lactic acid bacteria

The adhesion ability of the selected LAB strains was tested *in vitro* using Caco-2 cell line. The maximum of 10.48, 9.91, 8.6, and 8.11% of adhesion was observed in the isolate LAB33, LAB114, LAB557, and LAB56, respectively (Figure 2).

The antimicrobial property of LAB strains was tested against representative bacterial and fungal pathogens. The results were represented in Table 2. Almost, all the tested isolates were exhibiting antimicrobial property. LAB56, LAB67, and LAB68 showed higher activity against *S. aureus*, *B. cereus*, and *P. aeruginosa*. LAB354 and LAB709 showed potent antifungal activity against *C. albicans* (Table 2).

The generation time (min) and specific growth rate (μ) (h^{-1}) of the selected LAB strains were listed in Table 3. All the LAB strains are exhibiting the multiplication time anything between 50.06 and 60.12 min, and the specific growth rate of 0.67-0.83 h^{-1}. The fast generation time (50.06 min) and highest specific growth rate (0.83 h^{-1}) were observed in LAB33 strain (Table 3).

The immune activating property of LAB strains was assessed *in vitro* using Caco-2 cell line. The level of IL-6, and IL-10 were measured in Caco-2 cells after the exposure to LAB strains. IL-6 and IL-10 are the representative proinflammatory, and anti-inflammatory cytokines, respectively. About seven LAB strains induce the expression of IL-6, and only two isolates provoked the IL-10 expression *in vitro* (Figure 3). LAB33 induced the expression of IL-6 (0.76 ± 0.02 pg/mL) and IL-10 (262.34 ± 0.12 pg/mL) in Caco-2 cells. Likely, LAB114 induced the expression of IL-6 (~1.23 ± 0.01 pg/mL)

Table 2: The anti-microbial activity of isolated LAB strains

Strain code	*E. coli*	*S. aureus*	*B. cereus*	*P. aeruginosa*	*S. sonnei*	*S. Typhi*	*C. albicans*
LAB33	+	++	++	+	+	+	+
LAB56	++	+++	+++	++	+	++	+
LAB67	++	+++	+++	++	+	++	+
LAB68	++	+++	+++	++	+	++	+
LAB69	++	+++	++	+	+	++	+
LAB114	+	++	++	+	+	+	+
LAB238	++	++	++	+	+	++	+
LAB354	++	++	+	+	+	++	++
LAB512	++	++	++	+	+	+	+
LAB557	+++	+++	+++	++	+	++	+
LAB619	++	+++	+++	++	+	++	+
LAB709	+++	+++	+++	+	++	++	++

+++, ++, and +indicates the inhibition zone ≥10 mm, 7-9 mm, and <7 mm, respectively. LAB: Lactic acid bacteria, *E. coli: Escherichia coli, S. aureus: Staphylococcus aureus, B. cereus: Bacillus cereus, P. aeruginosa: Pseudomonas aeruginosa, S. sonnei: Shigella sonnei, S. Typhi: Salmonella Typhi, C. albicans: Candida albicans*

Table 3: The generation time and specific growth rate of selected lactic acid bacterial strains

Strain code	Generation time (min)	Specific growth rate (μ) (h^{-1})
LAB33	50.06	0.83
LAB56	55.32	0.75
LAB67	55.78	0.75
LAB68	53.88	0.77
LAB69	58.80	0.71
LAB114	52.14	0.81
LAB238	54.66	0.73
LAB354	55.20	0.77
LAB512	55.09	0.73
LAB557	60.12	0.67
LAB619	58.32	0.74
LAB709	54.87	0.72

LAB: Lactic acid bacteria

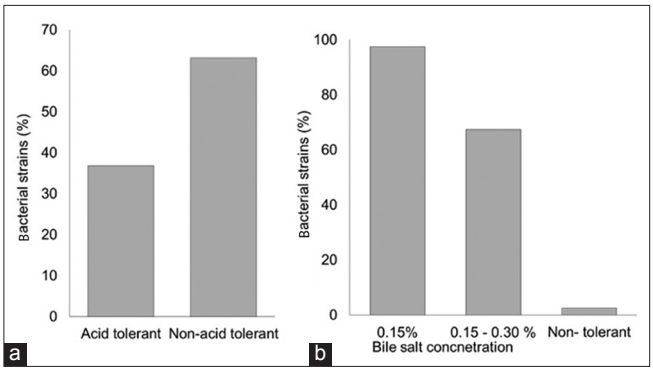

Figure 1: Acid tolerance (a) and bile salt tolerance (b) profile of isolated lactic acid bacteria strains. Total number of bacterial strains are represented in percentage (X-axis)

and IL-10 (255.46 ± 0.09 pg/mL) in Caco-2 cells. Whereas, other LAB strains are not able to induce the production of IL-10 (Figure 3).

The strains LAB33 and LAB114 were selected for the basic biochemical characterization based on the growth rate, generation time, and adhesion properties. The shape, cell wall nature, growth at high and low temperature, and sugar utilization properties were assessed (Table 4). Both strains are Gram-positive, rods, no catalase, no gas formation during glucose fermentation, and able to grow at 15°C. The difference was observed in the lactose, and raffinose utilizing property (Table 4).

Based on the results of screening and biochemical characterization, LAB33 was selected as a starter culture for the fermentation of soymilk (coded as FSM33). The LAB92 strain was used as control LAB strain, which has no immune activating property (coded as FSM92).

Soymilk fermentation processes were carried out as detailed. The pH, color, odor, taste, turbidity, and gas formation during beginning and end of the fermentation was recorded. The pH was found to be reduced from ~6 to ~3 in both FSM33 and FSM92. The color, smell, and taste of the soymilk became light brown, sour smell, and sour, respectively, in both FSM33 and FSM92, whereas the control sample (SM) was creamy yellow in color, soybean smell, and sweet during the fermentation. The phase separation and gas formation were also observed in both FSM33 and FSM92 samples, but not observed in SM (Table 5).

The microbiological safety assessment was performed for the FSM. The presence of coliforms, yeast and molds, *E. coli*, *Salmonella* sp., *S. aureus*, *C. perfringens*, and *B. aureus* was not found in both FSM33 and FSM92 after 30 days of fermentation. The total microbial count was 6.3×10^7, and 2.6×10^6 in FSM33, and FSM92, respectively. Whereas, no live microbes were detected in SM (non-starter control) (Table 6).

Anti-microbial activity of the FSM was evaluated and found that FSM33 and FSM92 have antimicrobial property against

E. coli, *S. aureus*, *B. cereus*, *P. aeruginosa*, and *C. albicans*. FSM33 was superior to FSM92 regarding antagonistic activity against *E. coli*, and *C. albicans* (Table 7).

LAB mediated fermentation process releases lactic acids in the medium. The level of lactic acid in the soymilk after 30 days of fermentation has been measured. FSM33 and FSM92 were detected with 0.87 ± 0.03, and 0.72 ± 0.05 g/100 mL lactic acid, respectively, indicating that LAB-mediated fermentation occurred in the samples. SM sample showed only 0.02 g/100 mL lactic acid after 30 days of fermentation because SM acted as a control (without starter culture). The results also suggested that the fermentation processes were carried out in an aseptic method, which supported by the microbial load calculation in SM sample (no microbes were detected) (Figure 4).

Table 4: Basic growth and carbohydrate utilization profile of LAB33 and LAB114

Character	LAB33	LAB114
Shape	Bacillus	Bacillus
Gram	+	+
Catalase	−	−
Gas from glucose	−	−
Growth at 45/15°C	±	±
Carbohydrates utilization		
Amygdalin	+	+
Arabinose	+	+
Cellobiose	+	+
Esculin	+	+
Fructose	+	+
Galactose	+	+
Glucose	+	+
Lactose	+	−
Maltose	+	+
Mannitol	+	+
Raffinose	−	+
Rhamnose	−	−
Ribose	+	+
Sorbitol	+	+
Sucrose	+	+
Trehalose	+	+

LAB: Lactic acid bacteria

The expression of IL-6 was found to be reduced (4.28 ± 0.03 pg/mL) in FSM33-treated cells compared to that of the SM (24.26 ± 0.04 pg/mL), and FSM92 (15.36 ± 0.13 pg/mL)-treated samples. Whereas, IL-10 expression was found to be significantly ($P < 0.05$) increased (282.13 ± 0.23 pg/mL) in FSM33-treated cells compared to that of the SM (47.45 ± 0.08 pg/mL), and FSM92 (118.21 ± 0.15 pg/mL)-treated samples. FSM92 exposure also significantly ($P < 0.05$) increased the expression of IL-10 compared to that of the SM (Figure 5).

DISCUSSION

The results suggested that most of the LAB strains used in the study are tolerant to bile salt at low concentration (Figure 1). The strong adhesion property of LAB is an important feature to act as a potent probiotic. Thereby, the probiotic strain can compete with other invading microbial pathogens for the niche in the host system. The results suggested that the LAB33 was the best isolate with a maximum percentage of cell adhesion (Figure 2). Based on the antimicrobial property, generation time, and immune activating property, LAB33 was selected for the fermentation of soymilk. The physical, biochemical, and health beneficial property of FSM was acceptable (Tables 5-7 and Figures 4 and 5).

A recent study demonstrated the use of mixed LAB strains with anti-inflammatory and antioxidant property to reduce the consequences of inflammation associated with colorectal cancer. In addition, the authors claimed that the combination of LAB strain supplementation help nullify the risk of development of colorectal cancer in patients with chronic intestinal inflammation.[23] Increased level of IL-10 was related to the reduced injuries and decreased

Table 5: The physical properties of LAB FSM

Time	Sample	pH	Physical property				
			Color	Smell	Taste	Turbidity	Gas formation
Day 0	SM	6.83	Creamy yellow	Mild soybean	Sweet	Slightly opaque	-
	FSM33	6.32	Creamy yellow	Mild soybean	Sweet	Slightly opaque	-
	FSM92	6.21	Creamy yellow	Mild soybean	Sweet	Slightly opaque	-
Day 30	SM	6.76	Creamy yellow	Mild soybean	Sweet	Slightly opaque	-
	FSM33	3.14	Light brown	Sour smell	Sour	Separation and sedimentation of creamy yellow. The upper part is quite transparent	Bubbles at the surface
	FSM92	3.46	Light brown	Sour smell	Sour	Separation and sedimentation of creamy yellow. The upper part is quite transparent	Bubbles at the surface

SM: Soymilk without starter culture, FSM33: Soymilk fermented with LAB33 as starter culture, FSM92: Soymilk fermented with LAB92 as starter culture. FSM: Fermented soymilk

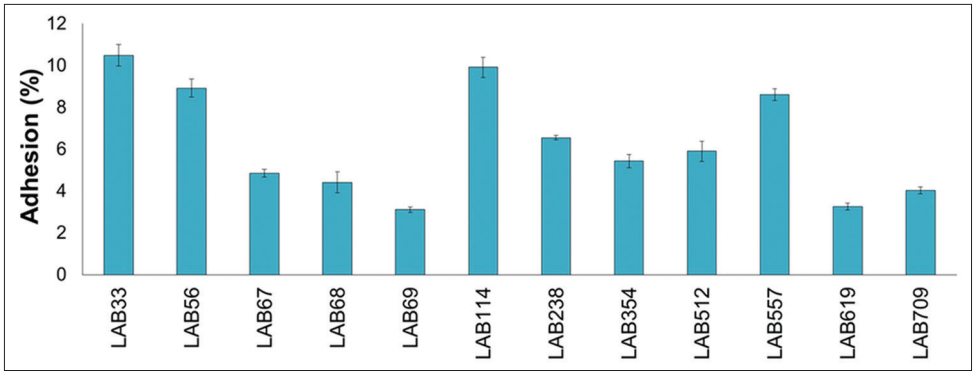

Figure 2: Percentage of adhesion of lactic acid bacteria strains on Caco-2 cells *in vitro*

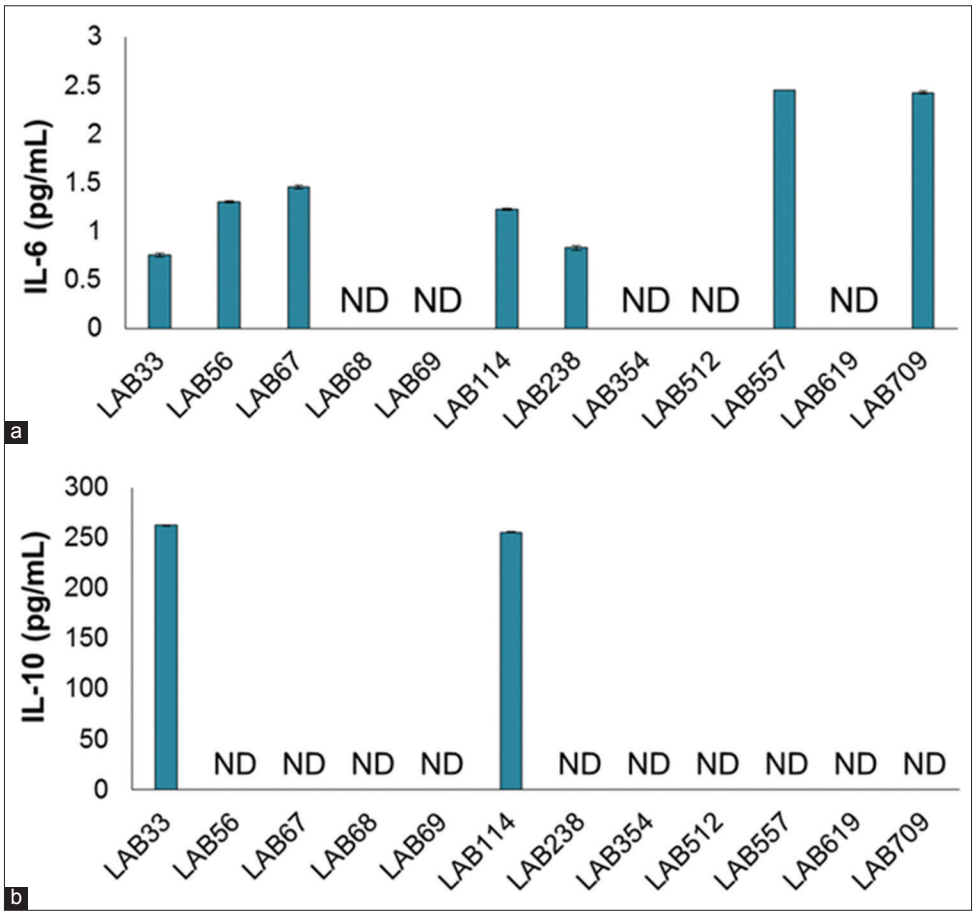

Figure 3: The level of expression of interleukin (IL) -6 (a) and IL-10 (b) in Caco-2 cells induced by lactic acid bacteria strains

Table 6: Microbial load in the FSM samples

Sample	Total microbes (CFU/mL)	Yeast and mold (CFU/mL)	Coliform bacteria (MPN/100 mL)	*E. coli* (CFU/mL)	*Salmonella* sp., *S. aureus, C. perfringens, B. cereus* (CFU/mL)
SM	Not found	Not found	0	0	Not found
FSM33	6.3×10^{7}	Not found	0	0	Not found
FSM92	2.6×10^{6}	Not found	0	0	Not found

SM: Soymilk without starter culture; FSM33: Soymilk fermented with LAB33 as starter culture; FSM92: Soymilk fermented with LAB92 as starter culture. *S. aureus: Staphylococcus aureus, B. cereus: Bacillus cereus, C. perfringens: Clostridium perfringens,* FSM: Fermented soymilk

levels of proinflammatory cytokines in the large intestine. Several *in vivo* studies and human trails revealed that IL-10 is involved in the maintenance of the intestinal immune homeostasis, but the oral supplementation of IL-10 is not

Table 7: The anti-microbial activity of fermented soybean milk

Sample	*E. coli*		*S. aureus*		*B. cereus*		*P. aeruginosa*		*C. albicans*	
	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30
SM	-	-	-	-	-	-	-	-	-	-
FSM33	-	+++	-	+++	-	+++	-	++	-	+++
FSM92	-	++	-	+++	-	+++	-	++	-	++

+++, ++, and +indicates the inhibition zone ≥20 mm, 11-19 mm, and <10 mm, respectively. Whereas, -: Indicates no activity.
E. coli: Escherichia coli, S. aureus: Staphylococcus aureus, B. cereus: Bacillus cereus, P. aeruginosa: Pseudomonas aeruginosa, C. albicans: Candida albicans, FSM: Fermented soymilk

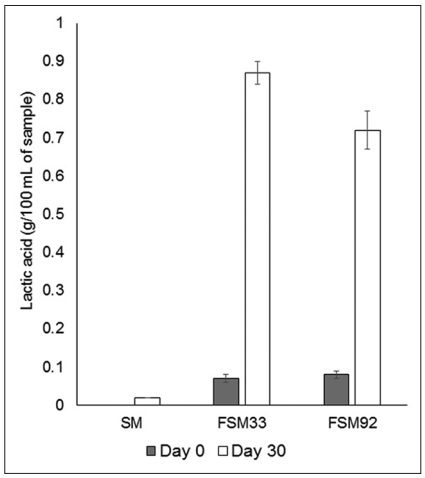

Figure 4: The lactic acid content of fermented soymilk samples. SM-soymilk without starter culture, FSM33-soymilk fermented with lactic acid bacteria (LAB) 33 as starter culture, FSM92-soymilk fermented with LAB92 as starter culture

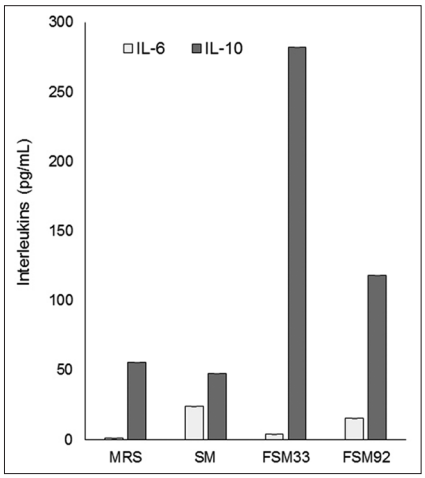

Figure 5: The level of interleukins released by the Caco-2 cells during the fermented soymilk (FSM)-mediated induction. MRS-Medium control, SM-soymilk without starter culture, FSM33-soymilk fermented with lactic acid bacteria (LAB) 33 as starter culture, FSM92-soymilk fermented with LAB92 as starter culture

effective because of its sensitivity to the gastrointestinal tract. Thus, IL-10 producing LAB strains were developed for the treatment of gastrointestinal tract discomfort and diseases. *In vivo* study using dextran sulfate sodium-induced colitis mice model revealed that *L. lactis* strain prevented the colitis, and proved that *L. lactis* is safe for human.[24,25] Some of the studies suggested that administration of LAB or IL-10 producing LAB mediated fermented food nullify the consequences of colitis in mice.[26,27] The oral supplementation of *Lactobacillus* HY7801 introverted the colon shortening and myeloperoxidase activity in 2, 4, 6-trinitrobenzenesulfonic acid-induced colitic mice, and decreased the expression of IL-1β, IL-6, and tumor necrosis factor-alpha,[28] which indicated that LAB could suppress the initiation or development of colitis. The upregulation of IL-10, an anti-inflammatory cytokine, reduces the symptoms of inflammatory bowel diseases (IBD). Dendritic cells, treg, and macrophages are major innate immune cells that produce IL-10.[29] The mechanism behind the increased level of IL-10 production upon LAB intervention is not clear. However, a recent study revealed that administration of *L. paracasei* LS2 increases CD4+FOXP3+ Treg cells, which are responsible for IL-10 production.[30] The results of the current study showed that LAB33 mediated FSM significantly induce the production of IL-10 and suppressed the IL-6 expression.

The present study explained the development of FSM using LAB33, which can modulate the immune system. The strain level identification of LAB33 is necessary to register the LAB33 based products in FDA as safe food supplements for human being.

The results suggested that the LAB33 is a strong microbial candidate for the development of several functional foods. The LAB33-mediated FSM can be used as dietary supplements for modulating the immune system of required people.

CONCLUSION

The isolated LAB33 was a potent isolate with the immune modulating property. Further, molecular identification of the strain is required. The LAB33-mediated FSM was considered as a functional food supplement to enrich the gastrointestinal immune system by inducing the expression of IL-10. The overall study revealed that LAB33 FSM as an alternative for the treatment of IBD.

ACKNOWLEDGMENT

BSS gratefully acknowledges the CMU Post-Doctoral Fellowship. Authors thankfully acknowledge the Faculty of Pharmacy, and Chiang Mai University, Thailand, for the support and instrumentation facilities.

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How to cite this article: Sirilun S, Chaiyasut C, Kesika P, Peerajan S, Sivamaruthi BS. Screening of lactic acid bacteria with immune modulating property and the production of lactic acid bacteria mediated fermented soymilk. Natl J Physiol Pharm Pharmacol 2017;7(12): 1397-1405.

Source of Support: Nil, **Conflict of Interest:** None declared.