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To evaluate the Effects of Lactic Acid Bacteria Fermented Lemon Juice from Limon and Eureka Varieties of Taiwan on Anti-pathogenic Bacteria and Anti-allergy

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Abstract Lemon juice contains many bioactive compounds that have potential health benefits. In this study, two different lactic acid bacteria (LAB) strains were used as starter cultures, change in pH, viable cell count during fermentation and antimicrobial, immune boosting, anti-allergy properties were monitored. LAB strains were able to grow in the juice and their viable cell reached to 8.0 log CFU/mL after 72 h at 30°C. Fermentation of lemon juice with LAB had a 2+ inhibitory ability against *Vibrio parahaemolyticus* and 3+ inhibitory ability against uropathogenic *Escherichia coli.* The inhibitory action of lactic acid fermentation against the pathogens may be due to the accumulation of main primary metabolites and pH changed during the fermentation. In the adhesion assay, PL LP10069 and TL PM229 exhibited stronger adherence to the Caco-2 cell line. With the anti-allergy test, the expression of anti-allergy cytokine of lemon juice fermentation in PBMC cell lines. The result indicated the significant enhanced to IL-12 by 1% NE PM229. The conclusion showed that fermentation of Lemon juice by probiotic bacteria not only increase antibacterial and anti-allergy effects but also enhance health benefit.

Keywords: *lemon, lactic acid bacteria, antimicrobial, anti-allergy*

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1. Introduction

Lemons contain many valuable natural products, such as nutrients, anticancer agents, aromatic compounds, and antioxidants. In recent years, *Lactobacillus* fermentation of food substrates has been found to improve the conversion of natural plant products and effectively increase their functionality, such as antioxidant, anticancer and anti-inflammatory properties [1]. Studies have reported that citral can inhibit pathogens such as *Escherichia coli*, *Salmonella typhimurium*, *Listeria monocytogenes*, and *Staphylococcus aureus.* Citral and linalool could effectively inhibit *Acinetobacter* spp., *Enterobacteriaceae* spp., *Moraxella* spp., and *Vibrionaceae* spp. isolated from the skin, gills, and intestines of fishes [2]. Addition of 5% dried lemon powder to beef meatballs reduced the growth of gram-positive bacteria and prevented the development of rancidity and peculiar aromas [3].

LAB can secrete antibacterial substances that inhibit gram-positive and gram-negative bacteria. Antibacterial substances produced via *Lactobacillus* fermentation such as lactic acid, acetic acid, and other organic acids show strong bacteriostatic effects against pathogenic bacteria [4,5]. Bacteriocins (proteins or peptides) produced by lactic acid bacteria show antibacterial activity and can be used as natural preservatives in foods such as cheese and processed cheese products [6,7]. Jay confirmed that diacetyl bacteriostatic substances inhibit *Bacillus* spp., and these substances are generally produced by *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., and *Streptococcus* spp [8]. The inhibitory effects of diacetyl substances on gram-negative bacteria, yeasts, and molds are superior to those on gram-positive bacteria [8].

LAB is gram positive. Therefore, studies have shown gram-positive bacteria induce the cytokine IL-12, which leads to Th1 immune responses. In contrast, gram-negative bacteria induce IL-10, which leads to Th2 immune responses [9]. The cell wall components of LAB exhibit immune-regulatory properties and contain peptidoglycans, polysaccharides, and teichoic acid [10]. Cell extracts from LAB induce morphological changes in macrophages and phagocytosis of macrophage pathogens. Moreover, lactic acid bacteria secrete peptides during milk fermentation, which increases IgA secretion and B-cell counts when fed to rats, improving immune function [11]. Furthermore, dead LAB can stimulate immune responses. Co-culture of

heat-killed *Bifidobacteria* with macrophages significantly increased TNF-α and IL-6 levels.

Citrus fruits contain hesperidin and flavanone glycosides, which possess anti-inflammatory and antiallergic activities [12]. A recent study found that metabolites produced by the *Lactobacillus* fermentation of lemons increased antibacterial activity and immunity as well as promoted the benefits of fruits to human body [1]. However, only a few previous studies have examined the anti-pathogenic bacteria and anti-allergic functions of the whole lemon fruit fermented by LAB. Therefore, the aim of this study was to examine the effects of hesperidin and flavanone glycosides from lemon skins on lactic acid bacteria fermentation. Initial screening was performed on the growth of lactobacilli in lemon juice. After identification using the API 50 CHL kit and based on 16S rDNA, antibacteria and anti-allergic activities of the fermentation broth were evaluated together with adsorption to intestinal epithelial cells and peripheral blood mononuclear cells (PBMCs).

2. Materials and Methods

2.1. Strain Culture

Lactobacillus strain PM229, which was antagonistic activity against urinary tract pathogen [13], isolated from vegetable pickles. Strain LP10069 was obtained from the Bioresource Collection and Research Center (BCRC). Lactobacilli stored at −80°C were activated twice. The culture medium used was MRS broth (DIFCO, Detroit, Michigan, USA) supplemented with 0.05% cysteine, and bacteria were cultured at 37°C for 20 hours.

2.2. Preparation of Lemon Fermentation Broth

Eureka and seedless (lime) lemon varieties were purchased from Taichung, Nantou, and Pingtung. Fresh lemons were washed with distilled water. After drying, the lemons were juiced, and a 0.149 mm mesh filter was used to filter off the peels. The juice was centrifuged at 6,080 ×g for 10 minutes, and the pH was adjusted to 3.5 using sodium bicarbonate. Next, inulin fiber or glucose was added to adjust the juice to 15° Brix. After heat processing at 90°C for 5 minutes, the juice was cooled. Lactobacilli were activated, followed by centrifugation at 6,080 ×g for 10 minutes. The pellet was resuspended in sterile water, and 2% (8 log CFU/ mL) lactobacilli were added to lemon juice for fermentation at 30°C for 72 hours. *Lactobacillus* strains PM229 and LP10069 were found to be the most suitable for fermentation through screening. Individual strains were added to 500 mL lemon juice for fermentation and stored at −80°C.

2.3. Identification of *Lactobacillus* Strains

The commercial ZR Fungal/Bacterial DNA Miniprep System Kit was used to extract DNA from lactobacilli. Next, 25 μL of PCR reaction mix was added to microcentrifuge tubes. This reaction mix contained 2 μL of 2.5 mM dNTP (N = A, T, G, and C), 2.5 μL of 10 × PCR buffer, 1 μL of Primer *L. pla* F (5'-TGATTGGTGCTTGCATCATG-3'), 1 μL of Primer *L. pla* R (5'-TGAACAGTTACTCTCAGATA-3'), 0.2 μL of Taq DNA polymerase, 16.3 μL of sterile deionized water, and 2 μL of target genomic DNA. Then the tubes were placed in a thermocycler for PCR. PCR condition was denaturation: 94°C/60 s, annealing: 58°C/60 s, and elongation: 72°C/60 s. PCR was performed 40 cycles. For analysis, 5 μL of PCR reaction product was subjected to 2.0% agarose gel electrophoresis in 0.5×TAE buffer. A UV box was used for observation before photography.

2.4. Pathogen Inhibition by Lemon Fermentation Broth

Pathogens included *Vibrio parahaemolyticus*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp., *Streptococcus mutans*, *Helicobacter pylori*, and urinary tract pathogens. Pathogens were diluted to 10^7 CFU/ mL and streaked on Petri dishes using sterile cotton buds. Holes of 9-mm diameter were punched on Petri dishes. Lemon fermentation broth (1 mL) was centrifuged at 6,080 ×g for 10 minutes, and 100 μL supernatant was added to holes on the Petri dishes. After 12 hours of culture, the zone of inhibition was measured [14,15].

The antibiotic susceptibility of Pathogenic bacteria was studied using the agar diffusion disk. Antibiotic disc such as ampicillin, chloramphenicol, gentamicin, erythromycin, kanamycin, neomycin, streptomycin, tetracycline, penicillin G, spiramycin and Mueller–Hinton agar were purchased from Difco (Detroit, Michigan, U.S.A.). Concentrations of the antibiotics were in accordance with the National Committee for Clinical Laboratory Standards (NCCLS) recommendations [16]. The antimicrobial activity was determined by measuring the clear zone around the disks after incubation at 37 C for 14 h.

2.5. Cell Culture

Human rectal cancer (CaCo-2) cell lines were purchased from BCRC and cultured according to the BCRC protocols. The method described by Prescott et al. was used for the isolation of mouse peripheral blood mononuclear cells [17]. Male ICR mice were purchased from BioLASCO Taiwan Co., Ltd (Taipei, Taiwan). Blood samples (8 mL) were added to cell preparation tubes (BD, USA), and the principle of Ficoll–Paque density gradient was employed. The tubes were centrifuged at 18°C and 1,500 ×g for 15 minutes. The first serum layer was removed, and peripheral blood mononuclear cells in the second layer were aspirated and collected into centrifuge tubes. Hank's buffered salt solution was added and gently mixed before the tubes were centrifuged at 18°C and 100 ×g for 10 minutes. The supernatant was discarded, and RPMI 1640 culture medium (supplemented with 10% FBS and 1% NEAA) was added. A hemocytometer was used to count cells. Cell concentration was adjusted to 2×10^6 cells/ well, and the cells were seeded in 24-well plates. Thereafter, the plates were cultured for 24 hours at 37°C and 5% CO_2 in an incubator.

2.6. Anti-allergic Effects of Lemon Fermentation Broth

PBMCs were seeded at a concentration of 2 × 10^6 cells/well, and different concentrations of lemon fermentation broth (1% and 2%) were added; culture medium alone was added to the blank control group, and OVA (200 μg/ mL) was added to the control group. The plates were cultured for 24 hours before subjecting to ELISA to quantify IFN-γ and IL-12 levels.

BD OptEIA™ kits (BD Biosciences, CA, USA) for human cytokines were used for cytokine quantification using ELISA according to the manufacturer's instructions. 96-well Immuno-Maxisorp plates (Nunc, Roskilde, Denmark) were coated with monoclonal antibodies, and placed in an incubating buffer overnight at 4°C. The plates were blocked and washed 3 times. Samples were added to the plates and incubated for 2 hours at room temperature. The plates were washed 3 times again, and biotinylated anti-human or anti-mouse along with horseradish peroxidase (HRP)-conjugated streptavidin were added for the detection of cytokines, and incubated 1 hour at room temperature. The reactions were developed using 3, 3′, 5, 5′-tetramethylbenzidine (TMB) substrate for 30 min at room temperature. The color reactions were stopped using 2N H_2SO_4 and absorbance was measured at 450 nm.

3. Results and Discussion

3.1. *Lactobacillus* Strains and Fermentation Conditions for Lemon Fermentation Broth

After Pingtung Eureka (PE) lemons were juiced, pH was adjusted to 3.5 and sugar content was adjusted to 15° Bx. After heat processing, 2% lactobacilli (8 log CFU/mL) were added for lemon juice fermentation at 30°C for 72 hours. PM229 and LP10069 strains were found to be most suitable for fermentation, and bacterial counts could be maintained at 7–8 log CFU/ mL after fermentation (Table 1). As the difference in *Lactobacillus* counts between lemon juices in which glucose or inulin was added was limited, glucose was selected as the carbon source for *Lactobacillus* fermentation due to cost considerations. After fermentation with different strains, the LAB counts of Taichung and Nantou Limon lemon fermentation broths were 7.04–8.02, and 5.80-7.10 log CFU/mL, respectively (Table 1). The *Lactobacillus* counts remained at 8.08–8.18 log CFU/ mL for Taichung Eureka fermentation Lemon juice and 7.99-8.79 log CFU/mL for Nantou Eureka (NE) fermentation Lemon juice (Table 1).

Table 1. The number of lactic acid bacteria (Log CFU/ mL) for lemon juice of different origins and varieties fermented by lactic acid bacteria at 30°C

Source	Variety	Log CUF/mL					
		Unfermented juice		PM229		LP10069	
		0hr	72hr	0hr	72hr	0hr	72hr
Taichung	Limon	0	0	6.91±0.01	8.02±0.09	6.90±0.02	7.04±0.06
	Eureka	0	0	6.96±0.02	8.18±0.10	6.68±0.05	8.08±0.02
Nantou	Limon	0	0	6.66±0.04	7.10±0.01	6.35±0.05	5.80±0.00
	Eureka	0	0	6.93±0.00	7.99±0.01	6.48±0.02	8.79±0.09

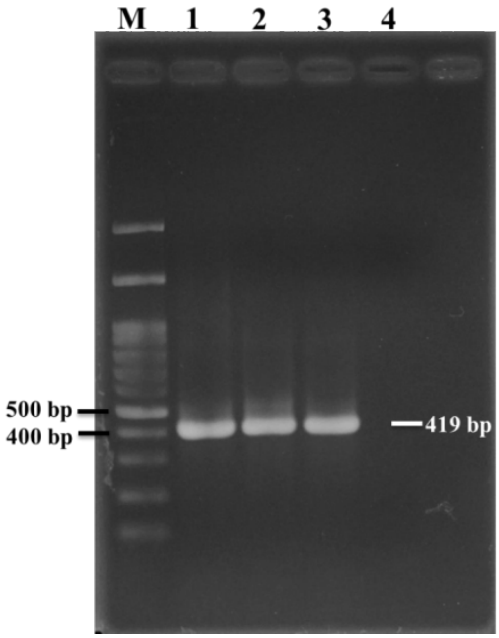

Figure 1. PCR detection of *L. plantarum* cells using PCR primers. Experimental conditions were as described in Methods. Lane M: 100 bp ladder. lane 1~3: PCR products amplified from 3 strains of LAB strain BCRC 12251(positive control), LP10069, PM229. lane 4: blank control without target cells

According to Islam et al., pH of lemon juice was adjusted to 2.0–7.0 before *Lactobacillus* fermentation [18]. In a study, our results showed that *Lactobacillus* growth was optimal at pH of 3.35–4.35 (Table 2), the *Lactobacillus* strains used in our experiments showed good growth in lemon juice. 16S rDNA PCR identification was performed; PM229 and LP10069 strains were identified as *L. plantarum* (Figure 1).

Table 2. The pH value of lemon juice from different origins and varieties after being fermented by lactic acid bacteria at 30°C

Source	Variety	pH of supernatant		
		Unfermented juice	PM229	LP10069
Taichung	Limon	3.44±0.06	3.49±0.01	3.47±0.04
	Eureka	3.50±0.01	3.45±0.05	3.69±0.02
Nantou	Limon	3.37±0.01	3.35±0.00	3.35±0.01
	Eureka	3.41±0.00	3.40±0.01	4.35±0.00

3.2. Antibacterial Activity of Lemon Fermentation Broth

The zone of inhibition experiment was used to examine the bacteriostatic activity of lemon fermentation broths (Table 3). TE and NL Lemon fermentation using PM229 strain exhibited 3+ inhibition of *Vibrio parahaemolyticus*,

similar to chloramphenicol and tetracycline (Table 4). TE, TL, NE and NL lemon fermentation by PM229 strain exhibited 2+ inhibition of *Streptococcus mutans* (Table 3), similar to Ampicillin, kanamycin and Spiramycin (Table 4). All lemon fermentation with PM229 and LP10069 exhibited 3+ inhibition of uropathogenic *Escherichia coli* (Table 3), similar to ampicillin, tetracycline and chloramphenicol (Table 4).

The bacteriostatic activity of lemon juice and lemon fermentation broth was hypothesized to be associated with pH and *Lactobacillus* metabolites, such as bacteriocin, hydrogen peroxide, and organic acids. Bacteriocins are small-molecular peptides produced by *Lactobacillus*, which are heat-stable and possess bacteriostatic activity, inhibiting the growth of *Escherichia coli*, *Streptococcus* spp., and *Pseudomonas* spp. [19]. Annuk et al. pointed out that hydrogen peroxide is a strong oxidizing agent that can disrupt the basic structure of proteins in microorganisms and attack cells to inhibit bacterial growth [20]. Gopal et al. showed that lactic acid and small-molecular peptides secreted by *Lactobacillus* show synergistic bacteriostatic effects [21].

3.3. Human Intestinal Epithelial Cell (Caco-2) Adhesion Test

Table 5 shows that the mean number of bacteria adhered to each cell were 44 bacteria for PM229 (44.7 ± 8.53 bacteria/cell). After fermentation of TL lemons, the adhesion of PM229 increased (47.1 ± 9.02 bacteria/ cell). LP10069 was 40 bacteria adhered to every cell on average (40.9 ± 9.78 bacteria/ cell). After fermentation of PL lemons, *Lactobacillus* adhesion was 41.8 ± 5.45 bacteria/cell.

Intestinal epithelial cells provide a natural barrier for the host to resist foreign substances. Upon entering the gut, microorganisms adhere to epithelial cells, which reduces invasion and adhesion of pathogens and promote host immune function [22]. Current studies have reported that factors facilitating *Lactobacillus* adhesion to epithelial cells include hydrophobic effects, lipoteichoic acids, lectins, and exopolysaccharides (EPA), among others [23,24,25]. EPAs are often attached to cell surfaces or secreted into the extracellular culture medium. A study reported that purification of EPA on the cell surface of *L. plantarum* significantly decreased adhesion and that adhesion was affected by trypsin treatment [26].

Table 3. The antibacterial ability of lemon juice, lemon fermentation broth and supernatant of lactic acid bacteria on pathogenic bacteria

Source	Variety	supernatant	Inhibition zone diameter (mm)						
			BCRC 12865[a]	BCRC 13829	ISM27	BCRC 13086	BCRC 15254	BCRC 15415	BCRC 10675
	MRS Broth	PM229	+(13)[b]	-	-	-	+(11)	+(13)	++(22)
		LP10069	+(12)	-	-	-	+(12)	+(12)	++(21)
Taichung	Eureka	Unfermented juice	+++(24)	++(17)	++(18)	+(15)	++(17)	+(16)	+++(28)
		PM229	+++(24)	+(16)	+(16)	+(16)	++(18)	+(16)	+++(30)
		LP10069	++(22)	+(15)	+(15)	+(15)	+(15)	+(14)	+++(28)
	Limon	Unfermented juice	++(22)	+(15)	+(14)	+(13)	++(18)	+(16)	+++(25)
		PM229	++(22)	+(14)	+(14)	+(14)	++(17)	+(16)	+++(28)
		LP10069	++(22)	+(14)	+(14)	+(13)	++(17)	+(16)	+++(27)
Nantou	Eureka	Unfermented juice	+++(23)	+(15)	+(15)	+(15)	++(17)	+(16)	+++(27)
		PM229	++(22)	+(15)	+(14)	+(15)	++(17)	+(15)	+++(28)
		LP10069	++(17)	+(14)	+(12)	+(14)	+(12)	+(14)	+++(27)
	Limon	Unfermented juice	+++(23)	+(15)	+(16)	+(15)	++(18)	+(16)	+++(28)
		PM229	+++(23)	+(15)	+(16)	+(15)	++(17)	+(16)	+++(27)
		LP10069	++(22)	+(16)	+(16)	+(15)	++(17)	+(16)	+++(27)

[a]BCRC 12865: Vibrio parahaemolyticus; BCRC13829: Staphylococcus aureus subsp.; ISM 27: Salmonella typhimurium; BCRC 13086: Escherichia coli; BCRC 15254: Streptococcus mutans ; BCRC 15415: Helicobacter pylori ; BCRC 10675: uropathogenic Escherichia coli.
[b]Interpretation of zone diameter of inhibition. –, less than 10 mm; +, 11–16 mm; ++, 17–22 mm and +++, more than 23 mm.

Table 4. The antibacterial ability of antibiotic disc on pathogenic bacteria

Antibiotic disc	Inhibition zone diameter (mm)						
	BCRC 12865	BCRC 13829	ISM 27	BCRC 13086	BCRC 15254	BCRC 15415	BCRC 10675
Ampicillin (10 μg)	-	-	-	++(20)	++(18)	++(19)	+++(24)
Kanamycin (30 μg)	+(15)	++(18)	++(19)	+(16)	++(21)	-	++(18)
Tetracycline (30 μg)	+++(25)	+(15)	+(15)	+++(25)	+(13)	+++(26)	+++(35)
Penicillin G (10 μg)	-	-	-	-	+(16)	-	-
Neomycin (30 μg)	+(15)	+(16)	+(16)	+(15)	-	+(13)	+(12)
Erythromycin (15μg)	+(14)	-	-	-	+++(23)	+(11)	+(11)
Streptomycin (10 μg)	-	-	-	+(14)	-	+(12)	++(18)
Gentamicin (30 μg)	++(19)	++(21)	+++(25)	++(18)	+++(24)	+(15)	+(16)
Chloramphenicol (30 μg)	+++(29)	-	-	+++(23)	+++(26)	+++(23)	+++(30)
Spiramycin (100 μg)	+(11)	-	-	+(11)	++(22)	+(12)	+(11)

[a]BCRC 12865: Vibrio parahaemolyticus; BCRC13829: Staphylococcus aureus subsp.; ISM 27: Salmonella typhimurium; BCRC 13086: Escherichia coli; BCRC 15254: Streptococcus mutans ; BCRC 15415: Helicobacter pylori ; BCRC 10675: uropathogenic Escherichia coli.
[b]Interpretation of zone diameter of inhibition. –, less than 10 mm; +, 11–16 mm; ++, 17–22 mm and +++, more than 23 mm.

Table 5. The effect of lemon juice from different origins fermented by lactic acid bacteria on the cell adhesion of human intestinal epithelial cells Caco-2

Source	Variety	Lactic acid bacteria	Adherence to the Caco-2
	MRS Broth	PM 229	44.7±8.53
		LP10069	38.9±9.62
Taichung	Eureka	PM 229	44.2±6.55
		LP10069	38.9±6.33
	Limon	PM 229	47.1±9.02
		LP10696	38.0±9.20
Nantou	Eureka	PM 229	35.0±2.31
		LP10069	35.5±9.30
	Limon	PM 229	39.7±7.79
		LP10069	38.9±7.55

The table presents mean number ± standard deviation of bacteria adhering per epithelial cell.

3.4. Effects of Lemon Fermentation Broth on PBMCs

In this experiment, lemon fermentation broth and stimuli (OVA) were used to treat PBMCs to evaluate the antiallergic activity of fermentation broth. Two percent lemon fermentation broth (TL PM 229, NL PM 229 and NL LP10069) can significantly stimulate PBMCs to secrete 1085.78 ± 7.66, 1308.26 ± 56.36 and 1262.93 ± 55.84pg/ mL IFN-γ, respectively (Figure 2). The amount of IFN-γ secreted by PBMCs treated with 1% lemon fermentation TL PM 229, TL 10696, NL PM229, or NL LP10069 was 1058.90 ± 45.67, 1101.02 ± 268.75, 1069.91 ± 76.32, or1129.35 ± 7.75 pg/ mL, respectively (Figure 2). The NE PM229 1% lemon fermentation broths significantly stimulated IL-12 secretion in PBMCs was 64.69 ± 14.81 pg/mL, respectively) (Figure 3).

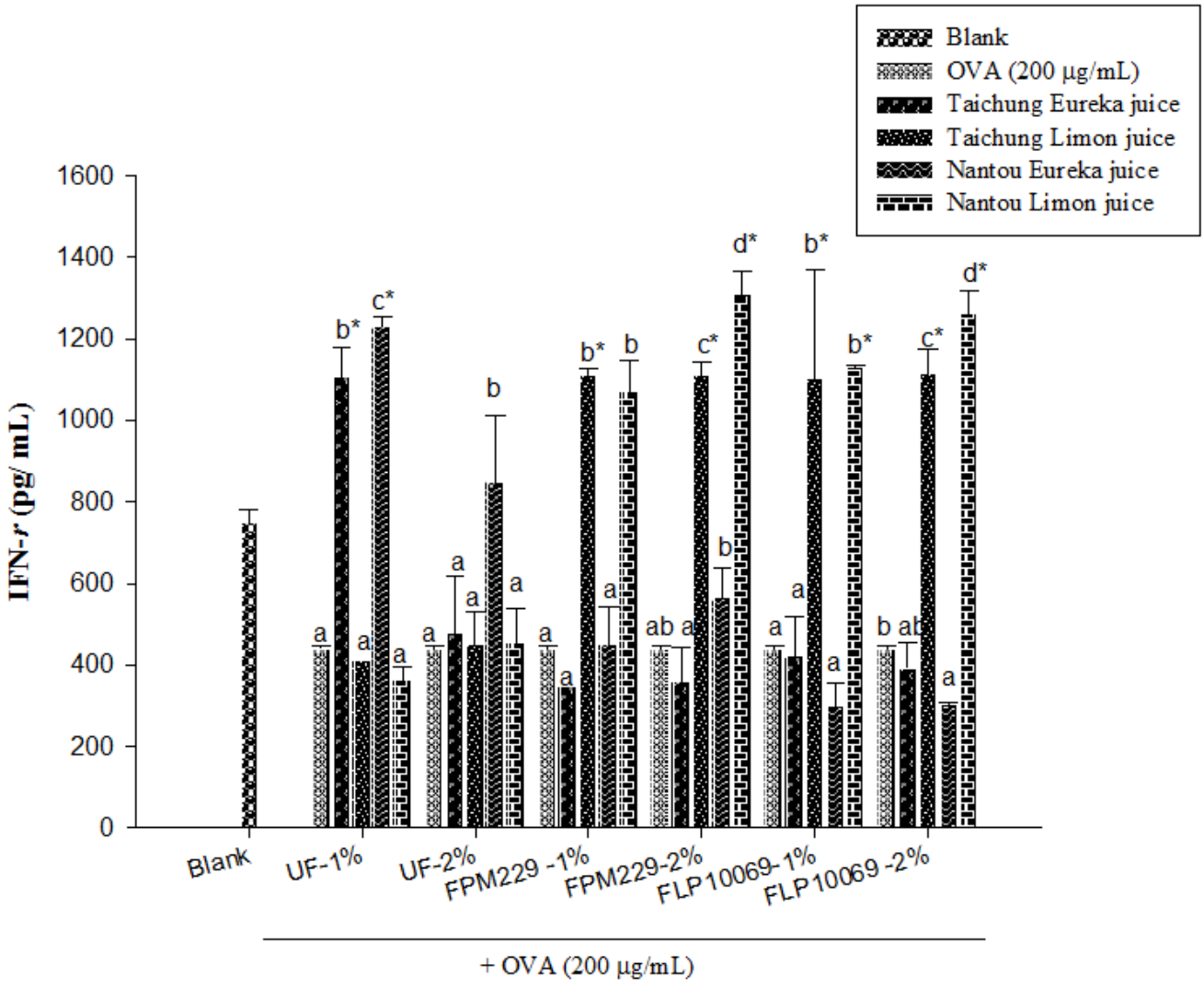

Figure 2. Effect of lemon fermentation by lactic acid bacteria on IFN- γ level secretion of PBMC cells induced with OVA. [a, b, c, d] Means with different superscript letters are significantly ($p<0.05$) different

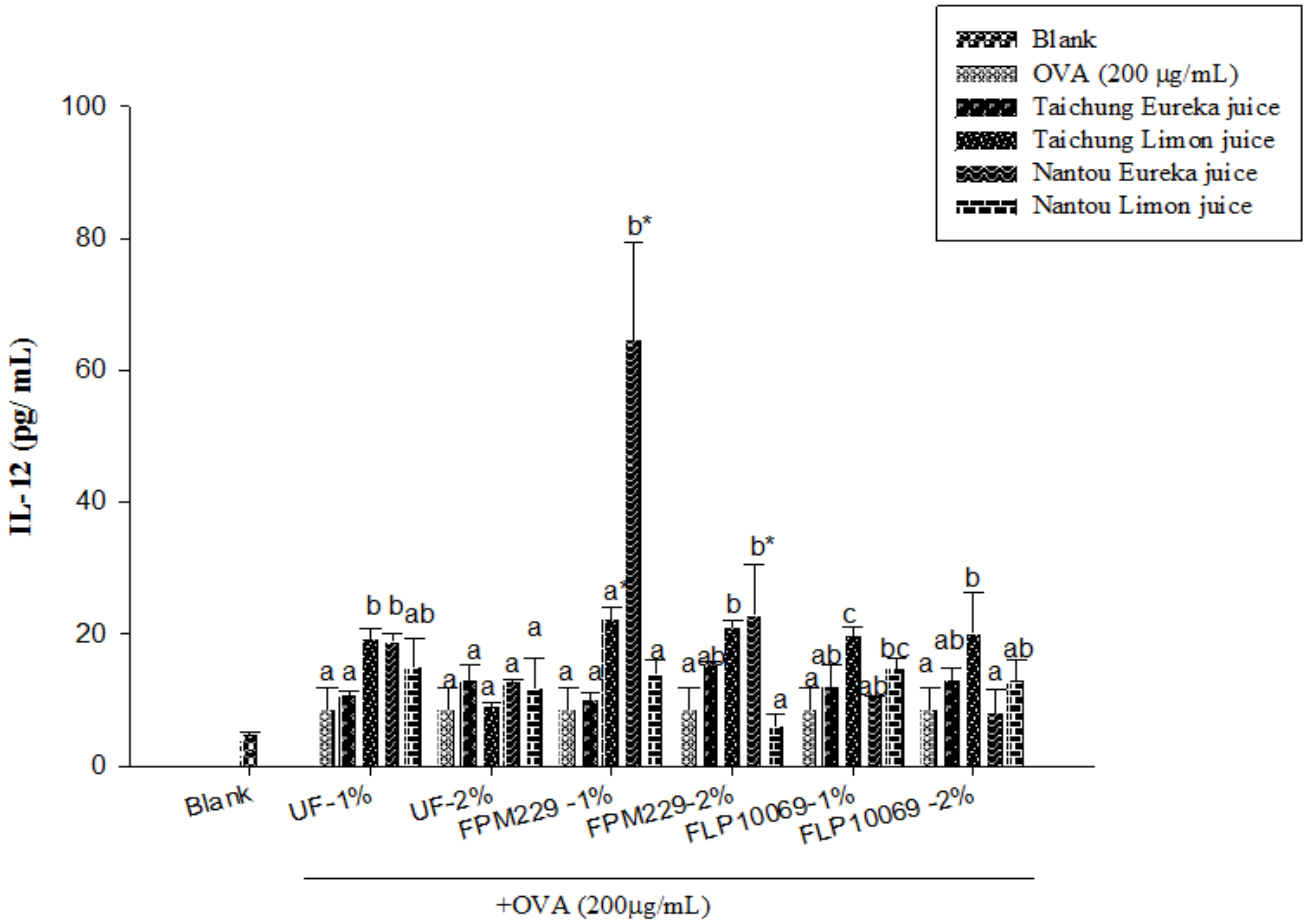

Figure 3. Effect of lemon fermentation by lactic acid bacteria on IL-12 level secretion of PBMC cells induced with OVA. [a, b, c] Means with different superscript letters are significantly ($p<0.05$) different

Most allergies are caused by the imbalance of immune secretion by lymphocytes and Th2 polarization by allergens. Th2 cells secrete IL-4, IL-5, and IL-13 to recruit eosinophils, basophils, and mast cells to the inflammation site [27,28]. Probiotics can adhere to intestinal cells in the host to improve intestinal mucosal permeability, increase intestinal IgA expression, elevate IL-10 and TGF-β secretion, and promote IFN-γ production to inhibit IL-4-induced IgE production hence reducing the occurrence of allergies [29]. PBMCs are composed of 60% lymphocytes and 16% monocytes [30,31]. A study in which *Lactobacillus casei* and OVA were added to mouse splenocytes *in vitro* showed that IL-12 regulated immune responses. This is because IL-12 is a potent inducer of IFN-γ and can stimulate cells to secrete IFN-γ and inhibit IL-4, IL-5, and IgE production [28]. Miettinen et al. co-cultured *Lactobacillus rhamnosus* E522 and E509 with PBMCs and found that mRNA expression levels of IFN-γ and IL-12 were significantly increased, these findings are similar to results of this study [32]. Additionally, Kekkonen et al. showed that *Streptococcus thermophilus* and *Leuconostoc* significantly induced IL-12 and IFN-γ secretion and switch Th2 immune responses to Th1 immune responses [33].

4. Conclusion

In summary, TL PM229 possesses antibacterial and the anti-allergic effects of Taichung limes were enhanced after PM229 fermentation. The total flavonoid content of TL PM229 was 4.37 mg/100 g, and its biological activity was significantly increased after fermentation. After lemon fermentation, PL LP10069 and TL PM229 fermentation broths increased *Lactobacillus* adhesion. Lemon contains compounds such as alkaloids, flavonoids, tannins, polyphenols and saponin. After being fermented by lactic acid bacteria, it has an additive effect on some functions such as immune regulation. Probiotic fermentation of lemon juice increased its health and nutritional benefits.

Declaration of Competing Interests

The authors have no competing interests.

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