

# Anti-Allergy Effect of Probiotic Goat Milk Yogurt with Indonesian Indigenous Bacteria *Streptococcus thermophilus* Dad-11 and *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13

Khariratun Horisah<sup>1</sup>, Dian Anggraini Suroto<sup>1</sup>, Kosuke Nishi<sup>2,3</sup>, Momoko Ishida<sup>2,3</sup>, Tyas Utami<sup>1</sup>, Yunika Mayangsari<sup>1</sup> and Takuya Sugahara<sup>2,3,\*</sup>

<sup>1</sup>Department of Food Science and Technology, Faculty of Agricultural Technology, Gadjah Mada University, Yogyakarta 55281, Indonesia

<sup>2</sup>Department of Bioscience, Graduate School of Agriculture, Ehime University, Ehime 7908566, Japan

<sup>3</sup>Food and Health Function Research Center, Ehime University, Ehime 7908566, Japan

(\*Corresponding author's e-mail: [sugahara.takuya.mz@ehime-u.ac.jp](mailto:sugahara.takuya.mz@ehime-u.ac.jp))

Received: 15 January 2025, Revised: 25 February 2025, Accepted: 4 March 2025, Published: 10 June 2025

## Abstract

Goat milk yogurt produced by an alternate culture of Indonesian Indigenous bacteria *S. thermophilus* Dad-11 and *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13 was made for the diversification of local goat milk products. The functionality of the product was evaluated to increase its value, which added to the probiotic properties. The study aimed to assess the anti-allergic properties of goat milk yogurt by identification of  $\beta$ -hexosaminidase release, intracellular  $\text{Ca}^{2+}$  concentration, microtubule formation, and western blotting in RBL-2H3 cells system. The GABA of goat milk yogurt was 97.9 ppm and no cytotoxicity was observed in the yogurt against RBL-2H3 cells. The yogurt water extract showed a more substantial anti-degranulation effect on RBL-2H3 cells than that of goat milk by suppressing the influx of  $\text{Ca}^{2+}$  into the cytosol and inhibiting microtubule formation. As further identification, the yogurt showed suppressive effects on the phosphorylation of Syk and PI3K, while slightly suppressing the phosphorylation of Akt and Lyn signaling pathways involved in degranulation. It can be concluded that goat milk yogurt fermented by *Streptococcus thermophilus* Dad-11 and *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13 have potential health benefits due to GABA content and anti-allergic effects.

**Keywords:** Yogurt, Goat milk, Anti-allergy, RBL-2H3 cells, Probiotic, *Lactiplantibacillus plantarum* subsp. *Plantarum* Dad-13, *Streptococcus thermophilus* Dad-11

## Introduction

Allergic diseases represent a growing global health problem. Allergy to milk products has become one of the most prevalent food allergies in young children, especially to cow milk. Approximately 2 - 6 % of babies exhibit signs of a cow's milk protein allergy [1]. This phenomenon poses a significant challenge as milk is a crucial nutrient needed for child growth, particularly for those who do not receive exclusive breastfeeding. Conversely, goat dairy products have gained popularity due to their lower allergenicity compared to cow milk. The appeal of goat dairy products is rooted in their

higher nutritional value, reported health benefits, and enhanced digestibility compared to cow milk [2,3].

Fermented food consumption also has been demonstrated to reduce or inhibit allergic responses through probiotic or fermenting microbial action. Fermented dairy products usually have particular lactic acid bacteria (LAB) that can produce various metabolites with health benefits, such as bioactive peptides and free amino acids [4]. Research has demonstrated that bioactive peptide production by LAB has immunomodulatory effects [5]. The utilization of LAB as probiotic bacteria in fermented dairy products

has the potential to hydrolyze antigenic proteins due to the increase of T cell activity, indicating a potential role in boosting immunological responses [6]. These processes are crucial for maintaining optimal immune responses and promoting human health. Some probiotic bacteria such as *Lactobacillus paracasei* subsp. *tolerans* JG22, *Bifidobacterium animalis* ssp. *lactis* HY8002, *Lactobacillus plantarum* HY7717 (HY7717), and *Lactobacillus sakei* K040706 (K040706) have been proven to show immunomodulatory activities [7 - 9]. The hypothesis suggests that the utilization of probiotic bacteria in goat milk-derived production has the potential to yield highly advantageous outcomes. This is attributed to the functional use of these products as a suitable substitute for cow's milk, which has been demonstrated to alleviate food allergies and gastrointestinal diseases [10].

*Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13 (formerly *Lactobacillus plantarum* Dad-13) is an indigenous lactic acid bacteria identified from traditional fermented buffalo milk, also known as "Dadih" in West Sumatra, Indonesia. This bacteria has been identified as a probiotic bacteria and has been applied as a yogurt starter co-culture with *Streptococcus thermophilus* Dad-11, resulting in comparable sensory profiles to commercial yogurt starter cultures [11,12]. However, lack of research in the utilization of *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13 and *Streptococcus thermophilus* Dad-11 as goat milk yogurt starters and the potential for anti-allergic activities. Although some LABs have been proven able to hydrolyze antigenic protein, their practical application must be evaluated on a strain-by-strain basis. Therefore, the current study investigated the anti-allergic activities of goat milk yogurt produced by *L. plantarum* Dad-13 and *S. thermophilus* Dad-11 on rat basophilic cell lines known as RBL-2H3 cells as compared with the raw material of yogurt.

## Materials and methods

### Microbial culture and reagents

*Streptococcus thermophilus* Dad-11 and *Lactiplantibacillus plantarum* subsp. *Plantarum* Dad-13, microorganisms utilized as initial fermentation cultures are sourced from the repository known as the Food and Nutrition Culture Collection (FNCC), Center for Food and Nutrition, Universitas Gadjah Mada,

Yogyakarta, Indonesia. Goat milk was purchased from Bhumi Nararya Farm.

### Probiotic goat milk yogurt production

#### *Fermentation of goat milk using indigenous starter cultures*

The fermentation was carried out according to [12] with modification. The freeze-dried starter of *L. plantarum* subsp. *plantarum* Dad-13 and *S. thermophilus* Dad-11 were incubated in goat milk with 37 °C for 24 h. Bacteria starters were prepared by inoculating 1 % cultures of *S. thermophilus* Dad-11 and *L. plantarum* subsp. *plantarum* Dad-13 (3:1, 10<sup>9</sup> CFU/mL) into goat milk. The goat milk yogurt and cell culture were further freeze-dried before analysis and kept at -35 °C.

#### *Lactic acid bacteria viable cell count*

The viable cell counts of *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13 and *Streptococcus thermophilus* Dad-11 in the yogurt samples were determined using a spread plate and plate pouring method based on [12]. The analysis was done by dissolving 5 mL of yogurt with 45 mL of 0.85 % NaCl, then followed by homogenization. A series of dilutions was made for each sample, a total of 0.1 mL of suspension was applied to the plated *Lactobacillus plantarum* Selective Media (LPSM) for enumeration of Dad-13, and a total of 1 mL of suspension was poured on de Mann Sharpe Rogosa (MRS) agar medium for total Lactic Acid Bacteria cell count. Agar plates were all incubated for 48 h at 37 °C. The number of colonies was measured and stated as log CFU/gram.

#### *Gamma Amino Butyric Acid (GABA) assay*

GABA quantification assay was carried out based on [13]. A cumulative volume of 1.5 mL of the specimen was subjected to centrifugation for 10 min at a rotational speed of 8,000 revolutions per minute within a 1.5 mL sample tube. After that, a 0.45 µm syringe filter was used to filter the supernatant. The cell-free supernatant was analyzed using HPLC (Shimadzu LC-10AD VP Liquid Chromatography, Kyoto, Japan). GABA was detected using a pre-column derivatization method with reagent *o*-phthaldialdehyde (OPA). 1 mL of methanolic OPA (0.01 g of OPA in 1 mL of methanol), 4 mL of borate buffer (pH 9) and 30 µL of 2-mercaptoethanol.

The derivatization was done by mixing 50  $\mu\text{L}$  of sample with 300  $\mu\text{L}$  of OPA solution. The mixture was homogenized for 2 min, then injected into the HPLC injector as much as 20  $\mu\text{L}$ . GABA was identified by comparing the retention time of GABA, and then the GABA concentration was calculated based on a comparison with the GABA standard curve.

### ***In vitro* study**

#### *Reagents*

Dulbecco's Modified Eagle Medium (DMEM), cell lysis buffer, mouse monoclonal immunoglobulin E specific to dinitrophenol (DNP), DNP-human serum albumin (HSA), bovine serum albumin (BSA), Triton X-100, and penicillin were procured from Sigma (St. Louis, MO, USA). A protease inhibitor cocktail, aprotinin, and Pefabloc® SC were obtained from Roche Applied Science (Basel, Switzerland). A phosphatase inhibitor cocktail was purchased from Nacalai Tesque (Kyoto, Japan). Goat anti-actin antibody and horseradish peroxidase (HSP)-labeled anti-goat IgG antibody, anti-Lyn, anti-phosphorylated Lyn, anti-phosphoinositide 3-kinase (PI3K) antibody, anti-phosphorylated PI3K antibody, anti-Syk, anti-phosphorylated Syk, anti-Akt, anti-phosphorylated Akt, and HRP-conjugated anti-rabbit immunoglobulin G antibody were sourced from Cell Signaling Technology (Danvers, MA, USA).

#### *Cell*

Rat basophilic cell lines, RBL-2H3 cells, were used for *in vitro* assay of the anti-allergy activity of probiotic goat milk yogurt water extract (PGYW).

#### *Sample extract preparation*

Probiotic goat milk yogurt, goat milk, Dad-13, and Dad-11 samples were extracted in distilled water for *in vitro* analysis. Each water extract sample was then named PGYW (probiotic goat milk water extract), GMW (goat milk water extract), D13W (Dad-13 water extract), and D11W (Dad-11 water extract). Freeze-dried samples were dissolved in distilled water, and then centrifuged at 10,000 rpm for 15 min at 10 °C. The supernatant was collected, neutralized, and freeze-dried. The dried samples were redissolved in sterile distilled water at 40 mg/mL. The water extracts were named as standard samples.

Probiotic goat milk yogurt water extract (PGYW) underwent a process of dialysis utilizing membranes characterized by molecular weight cut-offs of 14, 6.8, and 3.5 kDa, then freeze-dried and redissolved in distilled water at 40 mg/mL samples named as PGYD (probiotic goat milk yogurt dialysis extract). PGYW was also fractionated using 50 % ethanol. The ethanol-treated PGYW was assessed by separating the ethanol-soluble supernatant and the ethanol-insoluble precipitate. Both were freeze-dried and redissolved in distilled water at 40 mg/mL. The ethanol-treated fractions were named PGYES (probiotic goat milk yogurt ethanol supernatant extract) for the supernatant and PGYEP (probiotic goat milk yogurt ethanol precipitate extract) for the precipitate.

### **$\beta$ -Hexosaminidase release assay**

A modified version of the previously reported  $\beta$ -hexosaminidase release assay was utilized [14]. RBL-2H3 cells were cultivated for 12 h at 37 °C after being injected at a density of  $2.5 \times 10^5$  cells/well in a 24-well culture plate. After being cleaned with phosphate-buffered saline (PBS), 2 h were needed to activate the cells with 25 ng/well anti-DNP IgE. Following stimulation, the cells were twice washed with modified Tyrode's solution (MT buffer) to eliminate unbound anti-DNP IgE. Each well was then filled with MT buffer that contained bacterial lysate, goat milk, and probiotic goat milk yogurt aqueous extract. The wells were then incubated for 10 min, adding 100 ng/well of DNP-HSA; the mixture was incubated for half an hour. The culture supernatant was extracted from each well after 10 min chilled on ice to prevent the stimulation of antigen. The cells were sonicated to produce cell lysate after adding the cell lysis buffer.

$\beta$ -hexosaminidase release was determined by incubating cell lysate or culture supernatant with 0.1 M citrate buffer (pH 4.5) containing 3.3 mM p-nitrophenyl-2-acetoamide- $\beta$ -D-glucopyranoside at 37 °C for 25 min. The reaction was subsequently quenched with 2 M glycine buffer (pH 10.4), and absorbance at 405 nm was measured to quantify enzyme activity:

$$100 \times \frac{\text{Asupernatant} - \text{Ablank of supernatant}}{(\text{Asupernatant} - \text{Ablank of supernatant}) + (\text{Acell lysate} - \text{Ablank of cell lysate})}$$

### Measurement of intracellular $\text{Ca}^{2+}$ concentration ( $[\text{Ca}^{2+}]_i$ )

Intracellular calcium concentration ( $[\text{Ca}^{2+}]_i$ ) was monitored by fluorescent calcium indicator Fluo 3-AM (Dojindo Laboratories, Kumamoto, Japan), following previously established protocols [14,15]. A black 96-well culture plate was seeded with RBL-2H3 cells, and anti-DNP IgE was added. The cells underwent 2 PBS washes before being treated for 1 h with Fluo-3 AM as much as 100  $\mu\text{L}$ /well. PBS was used to rewash the treated cells once again before keeping the sample aqueous extract (20 mg/mL) at 37 °C incubation for 10 min. An SH-8000Lab microplate reader (Corona Electric, Ibaraki, Japan) was used to immediately measure the fluorescent intensity at an excitation wavelength of 490 nm and an emission wavelength of 530 nm after 10  $\mu\text{L}$  of DNP-HSA diluted with MT buffer at 0.625  $\mu\text{g}/\text{mL}$  was added to the cells to stimulate them.

### Immunofluorescent staining

A 35 mm tissue culture dish was seeded with  $4 \times 10^5$  RBL-2H3 cells/dish and 50 ng/mL anti-DNP IgE. The cells were then cultivated for 18 h at 37 °C. Cells sensitized to anti-DNP IgE were treated with 980  $\mu\text{L}$  of buffer that contained 20 mg/mL of each sample for 10 min at 37 °C following 2 buffer washes. After that, 20  $\mu\text{L}$  solution with 2.5  $\mu\text{g}/\text{mL}$  DNP-HSA in MT buffer dilution was used to activate the cells, and they were then incubated at 37 °C for 5 min. Following a PBS wash, the cells were fixed for 15 min at room temperature using 4 % paraformaldehyde. Following 3 5-minute PBS washes, PBS containing 0.3 % Triton X-100 and 5 % BSA was used to block the cell for 1 h. Cell Signaling Technology's Alexa Flour 488-labeled anti- $\alpha$ -tubulin antibody was used to stain the cells overnight at 4 °C using PBS containing 0.3 % Triton X-100 and 1 % BSA to observe tubulin. Following 3 5-minutes PBS washes, the cells were stained for 1 h at room temperature using an Alexa Flour 488-labeled anti-mouse IgG antibody diluted with PBS containing 0.3 % Triton X-100 and 1 % BSA. For fifteen minutes, the cells were exposed to DAPI solution diluted with PBS at room temperature following 3 rounds of 5-minute PBS washing. The cells were examined using a confocal microscope (Fluoview FV10i, Olympus, Tokyo, Japan) after being washed 3 times for 5 min with PBS and

treated with the anti-fading agent SlowFade (Molecular Probes, Eugene, OR, USA) to stop fading. The FV10-ASW program (Olympus) was used to analyze the pictures.

### Western blotting

The procedure for western blotting analysis was done as previously mentioned [14]. RBL-2H3 cells were treated with anti-DNP IgE after being plated at a density of  $2.5 \times 10^5$  cells/well into a 24-well culture plate. After washing the IgE-sensitized cells twice with MT buffer, each well was filled with 490  $\mu\text{L}$  of MT buffer containing sample aqueous extract (20 mg/mL), and the wells were incubated for 10 min at 37 °C. After that, DNP (10  $\mu\text{L}$ ) was added to activate the cell, diluted with MT buffer at 2.5 g/mL, and incubated for another 10 min at 37 °C. After removing the reagents, 30  $\mu\text{L}$  of lysis buffer containing 50 mM Tris, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 50 mM NaF, 30 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , 2 g/mL of aprotinin, and the phosphatase inhibitor cocktail were added to each well. The protein content of each supernatant was set at the same level after centrifuged for 15 min at  $12,000 \times g$  at 4 °C. After being separated by SDS-PAGE, the proteins in the cell lysate were put onto a Hybond-P PVDF membrane (GE Healthcare, Little Chalfont, UK). For 1 h at room temperature, 5 % skim milk dissolved in Tris-buffered saline with 0.1 % Tween 20 (TBS-T) was used to block the membrane. The membrane was cleaned with TBS-T and then incubated for the entire night with the primary antibody in 5 % BSA-TBS-T at 4 °C. After washing the membrane with TBS-T to get rid of the extra primary antibody, it was kept at room temperature for 1 h with HRP-labeled anti-goat IgG antibody or HRP-labeled anti-rabbit IgG antibody in 5 % skim milk-TBS-T. Blots were created using either the ImmunoStar LD reagent (Wako Pure Chemical Industries) or the ECL Western Blotting Detection Reagent (GE Healthcare) following washing with TBS-T to eliminate excess HRP-conjugated antibody. A ChemiDoc XPS Plus device (BioRad Laboratories, Inc. Hercules, CA, USA) was used to observe the bands, and Quantity One software (Bio-Rad Laboratories) was used to measure the chemiluminescent intensity.

## Statistics

The means  $\pm$  standard deviation (SD) express all results. ANOVA, or one-way analysis of variance, was performed using either the student t-test or Dunnett's test to assess statistical differences between groups. StatLight #4 software (Yukms, Tokyo, Japan) was used to do statistical studies.  $**p < 0.01$  or  $***p < 0.001$  were regarded as statistically significant, as were  $*p < 0.05$ .

## Results

### Probiotic Goat Milk Yogurt (PGY) production

#### pH and lactic acid bacteria after goat milk fermentation

Goat milk was fermented by *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13 mixed with *S. thermophilus* Dad-11 (3:1). After 36 h of fermentation, the pH was around 4.3 and at 37 °C, the starting LAB count for mixed cultures was roughly 9.48 Log CFU/mL. Meanwhile, for the count of *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13 was 8.07 Log CFU/mL and total lactic acid bacteria had 9.49 Log CFU/mL.

#### GABA production of PGY

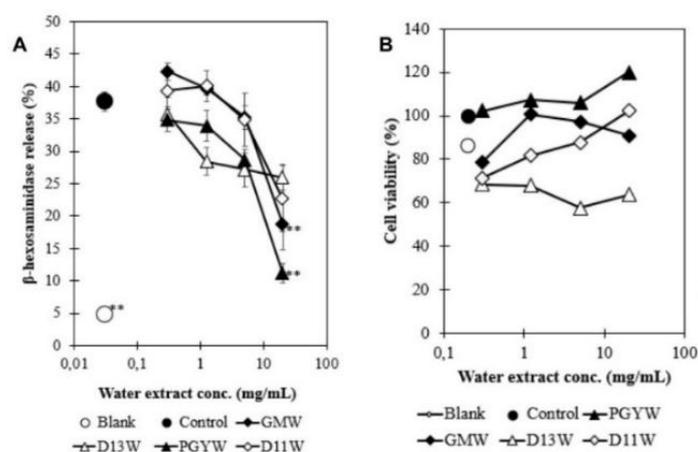
GABA (Gamma-aminobutyric acid) is a non-protein amino acid that functions as the central nervous system's main inhibitory neurotransmitter, enhances the production of immunity markers in humans, and regulates hormone secretion [16]. GABA was also discovered to inhibit mast cell and basophil activation,

suppressing degranulation by the GABA<sub>AB</sub> receptor [17]. The LAB fermentation process of milk has been known to increase GABA content. The production of GABA after fermentation was 97.9 ppm. The results showed a higher GABA concentration compared to goat milk fermented with other strains, including 12.84 mg/kg by *L. plantarum* 1,288 and 30.86 mg/kg by a combination of *L. plantarum* 1,288, *S. thermophilus* CR12, and *L. casei* LC01 [18].

### Anti-degranulation activity

#### Effect of standard samples on degranulation of RBL-2H3 cells

The anti-allergic potential of probiotic goat milk yogurt (PGYW) was evaluated by assessing its inhibitory effects on  $\beta$ -hexosaminidase release from RBL-2H3 cells. PGYW was applied at different concentrations to RBL-2H3 cells that were anti-DNP IgE-sensitized degranulated when stimulated with DNP-HSA as an antigen. Both PGYW and goat milk water extract (GMW) exhibited a dose-dependent inhibition of degranulation, however, PGYW demonstrated significantly greater potency. This observation suggests that PGYW contains higher concentrations of water-soluble compounds with anti-degranulation activity compared to GMW. Importantly, cytotoxicity assays (**Figure 1(B)**) revealed no detrimental effects of PGYW on RBL-2H3 cell viability. Based on these results, a concentration of 20 mg/mL PGYW was selected for subsequent experimental investigations.

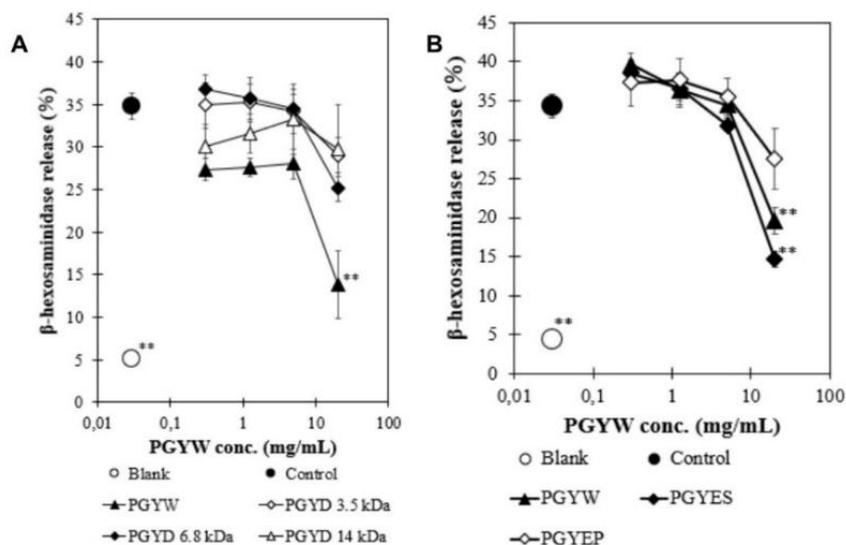


**Figure 1** Degranulation of standard samples and RBL-2H3 cells' viability. (A) Anti-DNP IgE-sensitized RBL-2H3 cells were exposed to varying amounts of standard samples for 10 min at 37 °C, and then DNP-HSA was used to cause degranulation. The released  $\beta$ -hexosaminidase was used to measure the degranulation. The data was displayed using the mean  $\pm$  SD (n = 3).  $**p < 0.01$  and  $*p < 0.05$  compared to the control. (B) Cell viability was determined using the WST-8 assay in anti-DNP IgE-sensitized RBL-2H3 cells stimulated with DNP-HSA in the presence of varying concentrations of standard samples or distilled water (DW) as control. Data represent mean  $\pm$  SD (n=3). N.S. indicates no significant difference.

### Effect of dialysis treatment and ethanol fractionation of PGYW on anti-degranulation activity

As shown in (Figure 2(A)), PGYD showed better activity than intact PGYW (standard sample). PGYW was also fractionated in EtOH-soluble supernatant and EtOH-insoluble precipitate by EtOH precipitation. The

result showed that the EtOH-soluble fraction (sup.; PGYES) of PGYW had higher activity than the EtOH-insoluble fraction (ppt.; PGYEP) as shown in (Figure 2(B)). These results suggested that the active component in PGYW possessing anti-degranulation activity was soluble in ethanol and had molecular weight lower than 3.5 kDa.

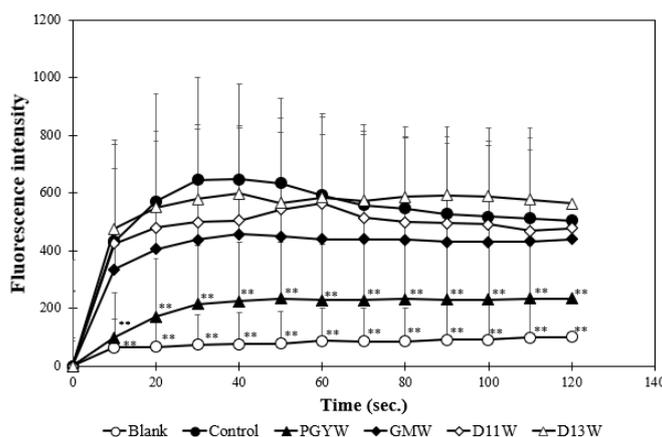


**Figure 2** Degranulation activity of dialysis treatment and ethanol fractionation against RBL-2H3 cells. (A) RBL-2H3 cells sensitized to anti-DNP IgE were exposed to several dialyzes or non-dialysis yogurt (PGYW) membranes. The mean  $\pm$  SD (n=3) was used to represent the data. \* $p < 0.05$ , \*\* $p < 0.01$  in comparison to the control. (B) RBL-2H3 cells sensitized to anti-DNP IgE were exposed to standard yogurt (PGYW) or ethanol fractionation. The mean  $\pm$  SD (n=3) was used to represent the data. \* $p < 0.05$ , \*\* $p < 0.01$  in comparison to the control.

### Effect of PGYW on intracellular calcium ion concentration ( $[Ca^{2+}]_i$ )

Degranulation occurs when the antigen attaches to mast cells or basophils' IgE, triggering intracellular signal transduction and raising the cells'  $Ca^{2+}$  content. The rise of intracellular  $Ca^{2+}$  levels ( $[Ca^{2+}]_i$ ) is a crucial degranulation process because it is a part of one of the many intracellular signaling second messengers [19]. Thus, the anti-degranulation effect of PGYW raw material on  $[Ca^{2+}]_i$  was examined using fluo-3 AM. This study analyzed 20 mg/mL of antibody-antigen-induced intracellular calcium ion concentration in RBL-2H3 cells.

PGYW suppressed the levels of  $[Ca^{2+}]_i$  more than other samples, as shown in (Figure 3), suggesting that by inhibiting the increase of  $[Ca^{2+}]_i$ , PGYW would reduce degranulation. GMW suppressed the  $\beta$ -hexosaminidase release in the degranulation assay. However, GMW didn't inhibit the increase of  $[Ca^{2+}]_i$ . This finding showed that the anti-degranulation effect of GMW was not along with the increase of  $[Ca^{2+}]_i$ . Meanwhile, bacteria water extracts did not suppress the elevation of  $[Ca^{2+}]_i$ , because they do not affect degranulation induced by antigen. This finding suggested that PGYW suppresses degranulation by inhibiting the intracellular signaling pathway that increases  $[Ca^{2+}]_i$ .

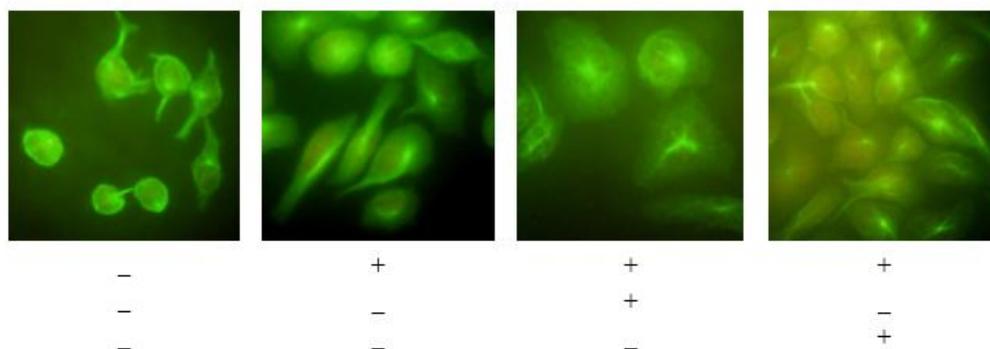


**Figure 3** Effect of standard samples on  $[Ca^{2+}]_i$  against RBL-2H3 cells. Intracellular calcium concentrations ( $[Ca^{2+}]_i$ ) were measured using the Calcium Kit-Fluo-3. **Blank:** Non-treated cells not stimulated with anti-DNP IgE. **Control:** Non-treated cells stimulated with anti-DNP IgE. **Samples:** Sample-treated cells stimulated with anti-DNP IgE. Data were represented as the mean  $\pm$  SD (n=3). \* $p < 0.05$ , \*\* $p < 0.01$  against control.

**Effect of standard samples on microtubule formation**

Microtubule formation is a critical step of degranulation in the calcium ion-independent pathway. As illustrated in (Figure 4), a thick and lengthy fibrous structure was elongated when the cells were activated by

antigen. Meanwhile, after being stimulated with GMW and PGYW, tubulin length was shorter, and there were fewer fibrous structures in the cells than in non-stimulated cells. The shorter tubulin with fewer threads indicates inhibition of microtubule growth.

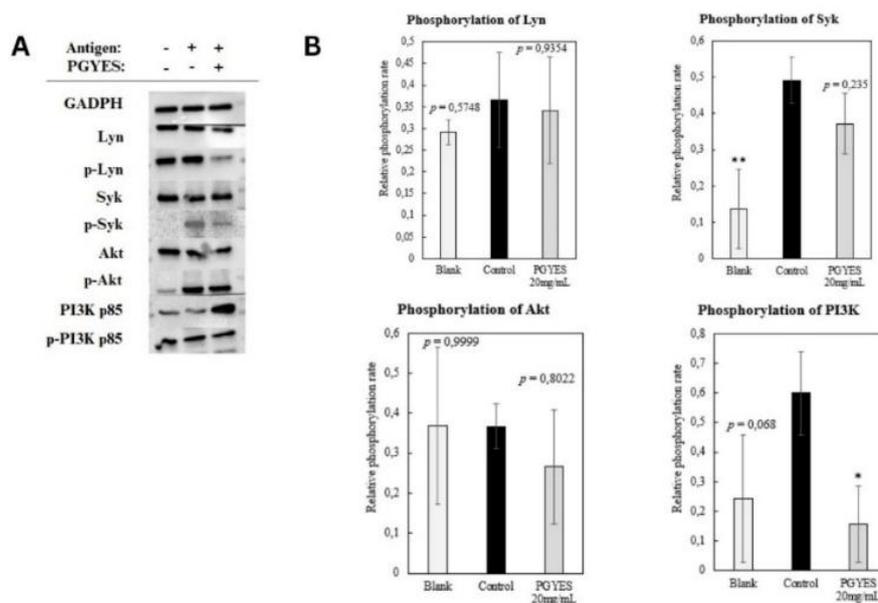


**Figure 4** The effect of yogurt and goat milk water extract on microtubule formation. 20 mg/mL of yogurt or goat milk was given to anti-DNP IgE-sensitized cells, and degranulation was obtained after 5 min of antigen stimulation. The cells were stained with an anti- $\alpha$ -tubulin antibody tagged with Alexa Fluor 488 and then examined under a confocal microscope.

**Effect of PGYES on signaling pathways involved in antigen-induced degranulation**

To assess the effect of the EtOH-soluble fraction of PGYW (PGYES) on the signaling pathways engaged in antigen-induced degranulation, western blotting or

known as immunoblot analysis was performed. PGYES was used because had higher anti-degranulation activity than PGYW. As seen in (Figure 5), PGYES inhibited the phosphorylation of Syk and PI3K while slightly inhibiting the phosphorylation of Lyn and Akt.



**Figure 5** Anti-degranulation pathways of RBL-2H3 cells by probiotic goat milk yogurt ethanol supernatant (PGYES): (A) Western blot analysis with specific antibodies of Syk with phosphorylated-Syk (p-Syk), Lyn with phosphorylated-Lyn (p-Lyn), PI3K with phosphorylated-PI3K (p-PI3K), and Akt with phosphorylated-Akt (p-Akt). GAPDH was used as a loading. (B) The ratio of p-Syk/Syk, p-Lyn/Lyn, p-PI3K/PI3K, and p-Akt/Akt in each cell lysate. Data were represented as the mean  $\pm$  SD (n=3). \* $p < 0.05$ , \*\* $p < 0.01$ , against control.

## Discussion

Yogurt is traditionally produced through the fermentation of milk by a combination of *S. thermophilus* and *Lb. bulgaricus* [20]. However, this study focuses on goat milk yogurt production using an alternate culture of Indigenous bacteria, *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13 and *Streptococcus thermophilus* Dad-11. Previous studies have shown that the combination of these bacteria can perform comparably to commercial starter cultures in cow milk [12]. To explore their additional capabilities, *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13 and *S. thermophilus* Dad-11 were used in goat milk yogurt fermentation. After 36 h of fermentation, the pH decreased to 4.3 because of acid production. The colloidal dispersion of casein micelles collapses when the pH turns into the isoelectric point of casein, resulting in acid casein precipitation and curd formation [21].

Since Dad-13 is a probiotic bacterium, the number of viable cells in the product is significant to ensure functionality. After fermentation, the number of Dad-13 fulfilled the minimum requirement for probiotics which is more than  $10^6$  CFU/g [22]. Based on these results, it

can be confirmed that goat milk yogurt fermented by Dad-13 and Dad-11 qualifies as a probiotic product. The health benefits of *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13 have been demonstrated through its ability to increase the population of *Lactiplantibacillus plantarum* subsp. *plantarum* in the feces of 30 healthy Indonesian people from 3 to 7 log CFU/g after consuming *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13 containing fermented drinks [11].

Moreover, goat milk yogurt produced by this bacterial combination exhibited notable GABA production capabilities. The results showed a higher GABA concentration compared to goat milk fermented with other strains, including 12.84 mg/kg by *L. plantarum* 1,288 and 30.86 mg/kg by a combination of *L. plantarum* 1288, *S. thermophilus* CR12, and *L. casei* LC01 respectively [18]. This finding suggested that the combination of *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13 and *S. thermophilus* Dad-11 demonstrated strong GABA-producing potential. Numerous studies have reported the GABA-producing abilities of lactic acid bacteria (LAB) strains in fermented dairy products [23]. This ability arises from GABA-synthesizing enzymes in LAB. Glutamic acid or

its salts play a crucial role in the conversion to GABA. Glutamic acid decarboxylase (GAD) catalyzes the irreversible  $\alpha$ -decarboxylation process of L-glutamic acid or its salts with the cofactor pyridoxal 5-phosphate (PLP; active vitamin B6) to produce GABA [24].

The health benefits of goat milk yogurt, PGYW are also investigated in terms of anti-allergic effects. Degranulation of cells was the primary sign of allergy mechanism. Histamine and  $\beta$ -hexosaminidase are 2 mediators released when RBL-2H3 cells degranulate. In this study,  $\beta$ -hexosaminidase was used as an indicator for the degranulation of RBL-2H3 cells due to its simplicity and speed detection. The bloodstream contains this mediator, leading to allergy symptoms, including runny nose, hives, and red, itchy eyes [25]. The result demonstrated that both goat milk yogurt and goat milk effectively suppressed  $\beta$ -hexosaminidase release, with yogurt exhibiting the most pronounced inhibitory effect. This finding suggests that the fermentation process of goat milk performs a pivotal function in the inhibition of degranulation, likely through the production of active substances. Furthermore, dialysis treatment and ethanol fractionation of the goat yogurt revealed the characteristics of the active substance present within it. The active substance in goat yogurt that contributed to suppressing degranulation was found to be ethanol soluble and had a molecular weight below 3.5 kDa. In skim milk, *Lactobacillus plantarum* has been shown to have capacity to produce capable of extracting 25 bioactive peptides with less than 3 kDa in molecular weight derived three 3 different protein types:  $\beta$ -casein,  $\alpha$ -S2-casein, and  $\alpha$ -lactalbumin [26]. Organic compounds with less than 3 kDa were also identified in *Lactobacillus plantarum* YE4 (YE4-CFE) extract, including adenine, acetylcholine, and L-phenylalanine that potential as anti-diabetic agents [27]. Moreover, *S. thermophilus* has been known to have a proteolytic system promoting the increase of peptides with a low molecular weight concentration released when proteins are hydrolyzed, most of which are less than 3.2 kDa [28].

The bacteria had no effect in suppressing  $\beta$ -hexosaminidase release, possibly because the bacteria were not in lysate form. However, while the bacteria themselves did not directly suppress the  $\beta$ -hexosaminidase release, they played an essential role in

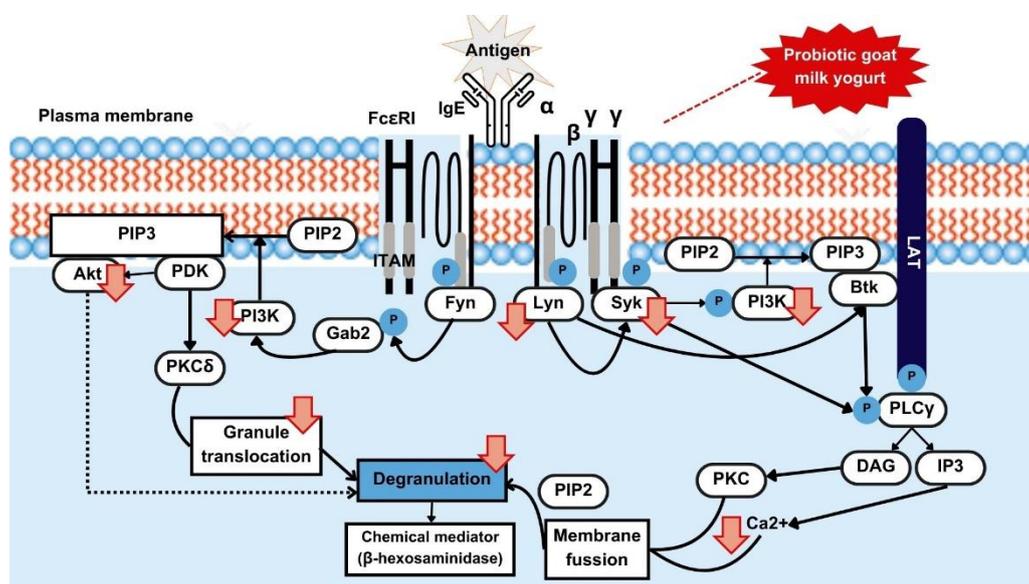
producing metabolites during fermentation that contributed to the inhibitory effect of yogurt on cell degranulation. The complex proteolytic system of lactic acid bacteria (LAB) is composed of peptidases, proteases, and a transport system [29]. Proteases hydrolyze milk proteins to produce polypeptides and amino acids during fermentation, destroying some epitopes and lowering the milk's antigenicity and allergenicity [30]. According to [31], administering yogurt T regulatory cell (Treg)-mediated inhibitory intestinal immune responses were enhanced in  $\alpha$ -CN+ $\beta$ -LG allergic mice when they were given milk instead. Similarly, skim milk fermented with *Lactobacillus casei* significantly lowered the ability of 4 essential milk proteins to bind IgE and their antigenicity:  $\alpha$ -LA,  $\beta$ -LG,  $\alpha$ -CN (including  $\alpha$ S1-CN and  $\alpha$ S2-CN), and  $\beta$ -CN [32].

The process of how probiotic goat milk yogurt inhibits degranulation was examined. When an antigen attaches to mast cells or basophils' IgE, intracellular signal transduction is started, raising  $[Ca^{2+}]_i$ . This process is known as degranulation. Mast cell degranulation has 2 pathways: The calcium-dependent Lyn-Syk-LAT pathway and the calcium-independent Fyn-Gab2-PI3K pathway [33,34]. The result showed that PGYW at a concentration of 20 mg/mL significantly suppressed intracellular calcium ion levels, while GMW did not. It suggests that PGYW inhibited degranulation by reducing the calcium ion concentration. Immunoblotting was used to measure the phosphorylation levels of the Syk and Lyn kinases to validate the results. Although not significant, the phosphorylation of Syk showed a suppressive effect. As members of the Src family of kinases, Lyn, Syk, and Fyn kinases are phosphorylated when antigens cross-link to Fc $\epsilon$ RI via IgE linkages, initiating an allergic reaction [35,36]. These receptors have subunits that contain sequences known as immunoreceptor tyrosine-based activation motifs (ITAMs) [37]. The  $\beta$ - and  $\gamma$ -chains of ITAMs are phosphorylated by activated Lyn kinase, and phosphorylation of  $\gamma$ -ITAMs triggers the activation of Syk kinase [38]. The phosphorylation of Syk occurs downstream of the Lyn kinase activity. In this study, Syk phosphorylation was suppressed by yogurt, while Lyn phosphorylation showed only a slight suppressive effect. Lyn is often used as a protein biomarker in the immunoblotting analysis because it is responsible for intracellular calcium ion mobilization, the degranulation

of mast cells and the activation of mitogen-activated protein kinases (MAPKs) [39]. Accordingly, yogurt's suppression of  $\gamma$ -ITAMs phosphorylation may be the cause of its prevention of Syk activation [40].

The ability of PGYW to inhibit degranulation was also observed in the calcium-independent pathways by inhibiting microtubule formation. In this experiment, GMW also appeared to inhibit microtubule formation but not to the same extent as yogurt. These results suggest that GMW can weakly inhibit degranulation via microtubule formation inhibition compared to PGYW. Cell degranulation results from microtubules facilitating granule translocation to the plasma membrane through

PI3K activation in the calcium-independent pathway [41]. Yogurt was shown to inhibit PI3K and Akt phosphorylation in the immunoblot analysis. PIP2 is phosphorylated to phosphatidylinositol trisphosphate (PIP3) by PI3K [42]. Moreover, Akt is activated by PIP3 as shown in (Figure 6), which is essential for Fc $\epsilon$ RI-induced degranulation [43]. According to these results, PGYW suppresses both calcium-dependent and calcium-independent mechanisms by downregulating the phosphorylation of Syk and PI3K while also slightly downregulating the phosphorylation of Lyn and Akt, all of which are involved in degranulation.



**Figure 6** The scheme of the anti-allergy effect of probiotic goat milk yogurt on RBL-2H3 cells.

## Conclusions

PGYW produced by fermentation with *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13 and *S. thermophilus* Dad-11 effectively prevent RBL-2H3 cells' degranulation in response to antigen without causing cytotoxic effects. The bacteria were also capable of increasing the GABA content in the yogurt, which contributes to reducing mast cell activation and degranulation. PGYW inhibited cell degranulation by suppressing  $[Ca^{2+}]_i$  influx and inhibiting microtubule formation. Further analysis revealed that the PGYW inhibits the phosphorylation of Syk and PI3K and slightly lowers the phosphorylation of Akt and Lyn, hence decreasing Fc $\epsilon$ RI-mediated intracellular signaling

pathways involved in the degranulation process. Thus, PGYW produced by fermentation with *L. plantarum* Dad-13 and *S. thermophilus* Dad-11 provides more excellent health benefits than unfermented GMW, particularly enhancing its anti-allergic effect.

## Acknowledgements

The authors thank to the Indonesia Endowment Fund for Education Agency (LPDP) and SUIJI-JP-Ms Program for financial support. The authors also thank Endang Sutriswati Rahayu for permitting to use the bacteria.

## References

- [1] D Lendvai-Emmert, V Emmert, A Makai, K Fusz, V Premusz, K Eklics, P Sarlos, P Toth, K Amrein and G Toth. Fecal calprotectin levels in pediatric cow's milk protein allergy. *Frontiers in Pediatrics* 2022; **9(10)**, 945212.
- [2] OI Guney. Consumer attitudes towards goat milk and goat milk products: A pilot survey in south east of turkey. *Turkish Journal of Agriculture - Food Science and Technology*. 2019; **7(2)**, 314.
- [3] TB Bedir and H Kuleasan. Determination of microbial properties of freeze dried traditional cheese. *Turkish Journal of Agriculture - Food Science and Technology* 2019; **7(4)**, 688.
- [4] AB Shori and AS Baba. Fermented milk derives bioactive peptides with antihypertensive effects. *Integrative Food, Nutrition and Metabolism* 2015; **2(3)**, 180-183.
- [5] B Foligne, S Parayre, R Cheddani, MH Famelart, MN Madec, C Plé, J Breton, J Dewulf, G Jan and SM Deutsch. Immunomodulation properties of multi-species fermented milks. *Food Microbiology* 2016; **53**, 60-69.
- [6] S Saito, A Okuno, Z Peng, DY Cao and NM Tsuji. Probiotic lactic acid bacteria promote anti-tumor immunity through enhanced major histocompatibility complex class I-restricted antigen presentation machinery in dendritic cells. *Frontiers in Immunology* 2024; **15**, 1335975.
- [7] JY Jung, JS Shin, SG Lee, YK Rhee, CW Cho, HD Hong and KT Lee. *Lactobacillus sakei* K040706 evokes immunostimulatory effects on macrophages through TLR 2-mediated activation. *International Immunopharmacology* 2015; **28(1)**, 88-96.
- [8] JY Kim, JY Kim, H Kim, EC Moon, K Heo, JJ Shim and JL Lee. Immunostimulatory effects of dairy probiotic strains *Bifidobacterium animalis* ssp. *lactis* HY8002 and *Lactobacillus plantarum* HY7717. *Journal of Animal Science and Technology* 2022; **64(6)**, 1117-1131.
- [9] SH Son, SJ Yang, HL Jeon, HS Yu, NK Lee, YS Park and HD Paik. Antioxidant and immunostimulatory effect of potential probiotic *Lactobacillus paraplantarum* SC61 isolated from Korean traditional fermented food, jangajji. *Microbial Pathogenesis* 2018; **125**, 486-492.
- [10] CS Ranadheera, CA Evans, MC Adams and SK Baines. Probiotic viability and physico-chemical and sensory properties of plain and stirred fruit yogurts made from goat's milk. *Food Chemistry* 2012; **135(3)**, 1411-1418.
- [11] ES Rahayu, MN Cahyanto, Mariyatun, MA Sarwoko, P Haryono and L Windiarti. Effects of consumption of fermented milk containing indigenous probiotic *Lactobacillus plantarum* dad-13 on the fecal microbiota of healthy Indonesian volunteers. *International Journal of Probiotics & Prebiotics*. 2016; **11(2)**, 99.
- [12] T Utami, A Cindarbhumu, MC Khuangga, ES Rahayu, MN Cahyanto, S Nurfiyanti and E Zulaichah. Preparation of Indigenous Lactic Acid Bacteria Starter Cultures for Large Scale Production of Fermented Milk. *Digital Press Life Sciences* 2020; **2**, 00010.
- [13] TW Kusuma. 2023, Produksi Asam Gamma-Aminobutirat (GABA) dari Isolat Lokal Bakteri Asam Laktat. Master Thesis. University of Gadjah Mada, Bulaksumur, Indonesia.
- [14] H Yoshioka, M Ishida, K Nishi, H Oda, H Toyohara and T Sugahara. Studies on anti-allergic activity of *Sargassum horneri* extract. *Journal of Functional Foods* 2014; **10**, 154-160.
- [15] Y Kitamura, K Nishi, M Ishida, S Nishimoto and T Sugahara. Anti-Allergic effect of aqueous extract of coriander (*Coriandrum sativum* L.) leaf in RBL-2H3 cells and cedar pollinosis model mice. *Nutraceuticals* 2022; **2(3)**, 170-180.
- [16] J Worarathphoka, S INNOK, P Soisungnoen, V Tanamool and W Soemphol.  $\gamma$ -Aminobutyric acid production and antioxidant activities in fresh cheese by *Lactobacillus plantarum* L10-11. *Food Science and Technology* 2021; **42**, 03121.
- [17] A Kawasaki, T Hara and T Joh. Inhibitory Effect of  $\gamma$ -Aminobutyric Acid (GABA) on histamine release from rat basophilic leukemia RBL-2H3 cells and rat peritoneal exudate cells. *Nippon Shokuhin Kagaku Kogaku Kaishi* 2014; **61(8)**, 362-366.
- [18] F Minervini, MT Bilancia, S Siragusa, M Gobbetti and F Caponio. Fermented goats' milk produced with selected multiple starters as a potentially

- functional food. *Food Microbiology* 2009; **26(6)**, 559-564.
- [19] R Cohen, DA Holowka and BA Baird. Real-time imaging of Ca(2+) mobilization and degranulation in mast cells. *Methods in Molecular Biology* 2015; **1220**, 347-363.
- [20] EFSA Panel on Dietetic Products and Nutrition and Allergies (NDA). Scientific opinion on the substantiation of health claims related to live yoghurt cultures and improved lactose digestion (ID 1143, 2976) pursuant to article 13(1) of Regulation (EC) No 1924/2006. *EFSA Journal* 2010; **8(10)**, 1763.
- [21] ER Vedamuthu. Starter cultures for yogurt and fermented milks. *Manufacturing Yogurt and Fermented Milks* 2006. <https://doi.org/10.1002/9780470277812.ch6>
- [22] T Vasiljevic and NP Shah. Probiotics-from metchnikoff to bioactives. *International Dairy Journal* 2008; **18(7)**, 714-728.
- [23] L Yu, X Han, S Chen, G Dunaan, S Feng, Y Xue, F Tian, J Zhao, H Zhang, Q Zhai and W Chen. Beneficial effect of GABA-rich fermented milk on insomnia involving regulation of gut microbiota. *Microbiological Research* 2020; **233**, 126409.
- [24] IBA Yogeswara, S Maneerat and D Haltrich. Glutamate decarboxylase from lactic acid bacteria-a key enzyme in GABA synthesis. *Microorganisms* 2020; **8(12)**, 1923.
- [25] A Elshemy and M Abobakr. Allergic reaction: Symptoms, diagnosis, treatment and management. *Journal of Scientific & Innovative Research* 2013; **2(1)**, 123-144.
- [26] Y Wang, B Zhao, Y Ding, N Liu, C Yang and Y Sun. Improved anti-oxidant and Anti-Bacterial capacities of skim milk fermented by *Lactobacillus plantarum*. *Molecules* 2024; **29(16)**, 3800.
- [27] J Sha, J Song, Y Huang, Y Zhang, H Wang, Y Zhang and H Suo. Inhibitory effect and potential mechanism of *lactobacillus plantarum* YE4 against dipeptidyl peptidase-4. *Foods* 2022; **11(1)**, 80.
- [28] JL Sebastian-Nicolas, E Contreras-Lopez, J Ramirez-Godinez, AE Cruz-Guerrero, GM Rodriguez-Serrano, J Anorve-Morga, J Jaimez-Ordaz, A Castaneda-Ovando, E Perez-Escalante, A Ayala-Nino and LG Gonzalez-Olivares. Milk Fermentation by *Lactocaseibacillus rhamnosus* GG and *Streptococcus thermophilus* SY-102: Proteolytic profile and ACE-Inhibitory activity. *Fermentation* 2021; **7(4)**, 215.
- [29] B Bianchi-Salvadori, P Camaschella and S Cislighi. Rapid enzymatic method for biotyping and control of lactic acid bacteria used in the production of yogurt and some cheeses. *International Journal of Food Microbiology* 1995; **27(2-3)**, 253-261.
- [30] C Bertrand-Harb, IV Ivanova, M Dalgalarrodo and T Haertle. Evolution of  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin content during yoghurt fermentation. *International Dairy Journal* 2003; **13(1)**, 39-45.
- [31] E Fuc, D Zlotkowska and B Wroblewska. Milk and Meat Allergens from Bos taurus  $\beta$ -Lactoglobulin,  $\alpha$ -Casein, and bovine serum albumin: An *in-vivo* study of the immune response in mice. *Nutrients* 2019; **11(9)**, 2095.
- [32] J Shi, Y Luo, Y Xiao, Z Li, Q Xu and M Yao. Effects of fermentation by *Lactobacillus casei* on the antigenicity and allergenicity of four bovine milk proteins. *International Dairy Journal* 2014; **35(1)**, 75-80.
- [33] RP Siraganian, ROD Castro, EA Barbu and J Zhang. Mast cell signaling: The role of protein tyrosine kinase Syk, its activation and screening methods for new pathway participants. *FEBS Lett* 2010; **584(24)**, 4933-4940.
- [34] R Sibilano, B Frossi and CE Pucillo. Mast cell activation: A complex interplay of positive and negative signaling pathway. *European Journal of Immunology* 2014; **44(9)**, 2558-2566.
- [35] WK Kanagy, C Cleyrat, M Fazel, SR Lucero, MP Bruchez, KA Lidke, BS Wilson and DS Lidke. Docking of Syk to Fc $\epsilon$ RI is enhanced by Lyn but limited in duration by SHIP1. *Molecular Biology of the Cell* 2022; **33(10)**, 89.
- [36] KH Sim, E Lee, P Shrestha, BH Choi, J Hong and YJ Lee. Isobavachin attenuates Fc $\epsilon$ RI-mediated inflammatory allergic responses by regulating SHP-1-dependent Fyn/Lyn/Syk/Lck signaling. *Biochemical Pharmacology* 2025; **232**, 116698.
- [37] J Zhong, N Tang, B Asadzadeh and W Yan. Measurement and correlation of solubility of

- theobromine, theophylline, and caffeine in water and organic solvents at various temperatures. *Journal of Chemical and Engineering Data* 2017; **62(9)**, 2570-2577.
- [38] S Kraft and N Novak. Fc receptors as determinants of allergic reactions. *Trends Immunol* 2006; **27(2)**, 88-95.
- [39] AM Gilfillan and J Rivera. The tyrosine kinase network regulating mast cell activation. *Immunological Reviews* 2009; **228(1)**, 149-169.
- [40] ROD Castro. Regulation and function of Syk tyrosine kinase in mast cell signaling and beyond. *Journal of Signal Transduction* 2011; **2011**, 507291.
- [41] K Nishida, S Yamasaki, Y Ito, K Kabu, K Hattori, T Tezuka, H Nishizumi, D Kitamura, R Goitsuka, RS Geha, T Yamamoto, T Yagi and T Hirano. FcεRI-mediated mast cell degranulation requires calcium-independent microtubule-dependent translocation of granules to the plasma membrane. *Journal of Cell Biology* 2005; **170(1)**, 115-126.
- [42] Y He, MM Sun, GG Zhang, J Yang, KS Chen, WW Xu and B Li. Targeting PI3K/Akt signal transduction for cancer therapy. *Signal Transduction and Targeted Therapy* 2021; **6**, 425.
- [43] Y Xu, D Nan, J Fan, JS Bogan and D Toomre. Optogenetic activation reveals distinct roles of PIP3 and Akt in adipocyte insulin action. *Journal of Cell Science* 2016; **129(10)**, 2085-2095.