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MORALITY BEFORE KNOWLEDGE

# Immunomodulatory effects of yogurt as a functional food: Enhancing innate immunity against influenza virus infection through in vivo analysis

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## ABSTRACT

**Background:** Influenza is a severe respiratory illness that affects people practically everywhere and is brought on by influenza viruses. Although vaccination is often used to prevent influenza virus infection, it is not always effective due to the fast rate of viral mutagenesis and the annual shift in the dominant virus strains. Boosting nonspecific innate immunity is another method of preventing influenza virus infection by strengthening natural defenses. Yogurt and other fermented functional foods have been shown to contribute to health maintenance. **Methods:** The objective of this review of these studies is to investigate and comprehend the in vivo approach to testing yogurt—a functional food—as an immunomodulator, particularly in influenza infection. **Findings:** Because extracellular polysaccharides in yogurt influence the immune system and provide protection against IAV infection, it does have immunomodulatory qualities. Yogurt eating on a daily basis before to infection significantly enhanced the survival rate of mice, the cytokine response in the lungs, and NK cell activity. It has been demonstrated that the ingredients in yogurt cause mice's splenocytes to produce more IFN-γ. **Conclusion:** Yogurt supplemented with nF1 has preventive and protective effects against IAV infection and can trigger immune responses by enhancing NK cell activity. **Novelty/Originality of This Study:** The novelty of this study lies in its exploration of yogurt as a functional immunomodulator, highlighting its role in enhancing innate immunity and providing protection against influenza virus infection through in vivo analysis.

**KEYWORDS**: functional food; immunomodulator; yoghurt.

## 1. Introduction

Influenza is an acute respiratory infection caused by influenza viruses that occurs almost worldwide, especially influenza A virus (IAV) infection which has become a global public health problem. Influenza A virus causes respiratory diseases or disorders ranging from mild to severe which tends to have high morbidity and mortality, depending on the virus strain and the host's immune competence (Taubenberger & Morens 2010). In particular, the elderly and young children are at greater risk due to weak immune function.

Vaccination is usually used to prevent influenza virus infection but is not necessarily sufficient because viral mutagenesis occurs rapidly, and the dominant virus strains change every year. Therefore, strengthen natural defenses is highly recommended by increasing nonspecific innate immunity in everyday life. The innate immune system will act as the front

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line of defense against IAV infection (Kreijtz et al., 2011). Natural killer (NK) cells play an important role in the defense against IAV infection by killing infected cells and producing cytokines as part of the innate immune response that can reduce IAV infection and develop an adaptive immune response (Schultz-Cherry, 2014) so that modulation of NK cell activity can increase immunity to IAV infection.

Fermented foods including lactic acid bacteria (LAB) and their products have been known to have a role in maintaining health since ancient times. Yogurt is one of the fermented products by LAB, especially Streptococcus thermophilus or Lactobacillus delbrueckii, with or without additional cultures (Alimentarius, 2010 in Kim DaHyun et al., 2018) with milk as the base ingredient. Several strains of L. plantarum have also been reported to provide protective effects against influenza viruses (Arimori et al., 2012; Kikuchi et al., 2014). In this study, oral administration of different yogurts was evaluated for their effects on influenza virus infection in mice.

Several studies have shown that yogurt consumption is effective for influenza virus infection because NK cells, which are lymphocytes from the innate immune system, play an important role in initial defense against various viral infections. Both studies aimed to test whether yogurt treatment has immunomodulatory properties, including NK cell activity. The effect of yogurt administration on preventing influenza virus infection in mice infected with IAV at a lethal dose was also evaluated. The study of this method itself aims to study and understand the in vivo method in testing functional food in the form of yogurt as an immunomodulator, especially in influenza infection.

### *1.1 Yogurt consuption*

Yogurt is a fermented milk product that can supply digestible lactose, live bacterial strains, mainly *Streptococcus thermophilus* and *Lactobacillus bulgaricus.* Yogurt has been consumed for a long time and is known in various parts of the world with distinctive names in each region. Yogurt is believed to have been present as human food consumption since 10-5 thousand years before century which is made from domestic livestock's milk, including cows, goats, sheep, buffalo, and camels (Fisberg & Machado 2015). Milk as a perishable good made it difficult to consume so at that time, people in the Middle East brought milk by placing it in containers made of intestines. Furthermore, it is known that the fluid from the digestive tract made the milk thick and sour, preserving it, so that it could be consumed for a longer time (Fisberg & Machado 2015).

Currently, commercially produced yogurt comes in variations with the addition of composition, flavours, and food additives. The types of yogurt also vary, such as whole milk, low-fat, non-fat, with plain or fruit flavours and other flavours, with the addition of natural or artificial sweeteners. The popularity of yogurt strains, or commonly known as Greek yoghurt or labneh, with a thicker consistency is also increasing (El-Abbadi et al., 2014). It can be consumed directly as a drink, or added to fruit mixture.

### *1.2 Yogurt nutrition*

Yogurt is one of the sources of various essential nutrients, such as protein, calcium, potassium, phosphorus, also B2 and B12 vitamins (Bodot & Soustre, 2013). According to Indonesian Food Composition Table Data (2017), fresh yogurt per 100 g has an energy content of 52 kcal, 3.3 grams of protein, 2.5 grams of fat, 4.0 grams of carbohydrates, and 2 grams of ash. Micronutrients contained in fresh yogurt consist of vitamin A as much as 22 mcg, vitamin B1 0.04 mg, B2 vitamin 0.1 mg, niacin 0.2 mg, Fe 0.1 mg, phosphorus 90 mg, natrium 40 mg, potassium 299 mg, copper 0.01 mg, zinc 0.6 mg, β-carotene 10 mcg, and calcium 120 mg. Probiotics and bioactive compounds produced during fermentation contained can improve health through their physiological effects. Yogurt can also affect diet with better changes in food consumption and improve the quality of daily food consumption. Many studies have analysed the contribution of yogurt to nutrient intake. The results showed that yogurt is able to improve metabolic profiles, especially the decrease

triglycerides in plasma and insulin concentrations (Cormier et al., 2016; Wang et al., 2013; Zhu et al., 2015). Consuming lactic acid bacteria (LAB) or its fermented products can also reduce the risk of infection and increase anti-influenza activity (Kawahara et al., 2015; Makino et al., 2016; Meng et al., 2016).

### *1.3 Yogurt parameter as an immunomodulator*

According to research, yogurt can modulate immune function and provide anti-influenza virus effects in mice, one of which is by increasing NK cell activity (Makino et al., 2016; Nishimura et al., 2016; Shida et al., 2017). With oral administration, *Lactobacillus casei* and *L. plantarum* showed a longer survival time after influenza virus infection and increased innate immunity. In these two studies, different lactic acid bacteria were used in the treatment. *Lactobacillus delbrueckii* ssp. bulgaricus (*L. bulgaricus*) OLL1073R-1 was derived from traditional Bulgarian yogurt and was said to produce exopolysaccharide immunostimulant (EPS) while *Lactobacillus plantarum* nF1 (nF1) is a LAB isolated from *kimchi*, a traditional Korean dish made using fermented cabbage that proven to have beneficial effects on the immune system (Choi et al., 2018).

#### *1.3.1 Viral titer*

Viral titer, can also be called viral load, is the quantity of virus present in a unit. Its value describes the amount of virus present in the blood, which is defined as the number of copies per milliliter (CDC, 2018). Plaque assays are a standard method that has been used to determine viral titer or infectious dose. This method can determine the number of plaque units formed or plaque forming units (PFU) in a sample. The PFU/mL result indicates the number of infectious particles in the sample, assuming each plaque is caused by one infectious virus particle (Ryu, 2017).

#### *1.3.2 Immunoglobulin*

Immunoglobulin (Ig) or antibodies are glycoproteins produced by plasma cells. B cells are ordered by specific immunogens, such as proteins in bacteria, to differentiate into plasma cells, which are cells that produce proteins that participate in providing humoral immune responses to bacteria, viruses, fungi, parasites, cellular antigens, chemicals, and synthetic substances. There are 5 types of immunoglobulins in humans, but those used as parameters in the study of the effect of yogurt on influenza infection are IgA and IgG (Vaillant et al., 2020).

IgG is a monomer that is mostly synthesized as a secondary immune response to pathogens. IgG can activate the traditional pathway of the complement system, and is also very protective. IgA has 2 different molecular structures, namely monomers (serum) and dimers (secretory). As the main antibody in the secretion system, IgA can be found in saliva, tears, colostrum, intestines, genital tract, and respiratory secretions. IgA has a secretory component that prevents its enzymatic digestion and can activate alternative pathways of complement system activation (Vaillant et al., 2020).

#### *1.3.3 Natural killer cell activity*

Natural killer cell or NK cell activity is a commonly used parameter in determining the performance of lysis activity against tumors and virus-infected cells in innate immunity. However, the use of this parameter has several constraints related to bioassay measurements. To measure NK cell activity, target cells and cell culture equipment are needed and the importance of pre-culture of target cells ensures the availability of pre-cultured NK cells so that cell sensitivity remains constant. NK cell activation receptors play an important role in recognizing targets, which transduce the necessary signals so that they can induce target injury and cytokine production (Nishimura et al., 2017).

### *1.3.4 Gene and messenger RNA expression*

Gene expression describes the phenotype manifestation of genes based on the process of gene transcription and translation. Gene expression requires the synthesis of various RNAs, including mRNA or protein-coding messenger RNA, rRNA or ribosomal RNA, and tRNA or transfer RNA (Jun et al., 2013). This is very important in maintaining normal cell structure and function. The ability of gene expression to regulate allows cells to supply functional proteins whenever the body needs them to function and stay alive (Verma & Singh, 2013). The technology that can be used to analyse it is by evaluating the effect of increasing immunity from the treatment given. Mice were given yogurt orally every day and their organs were tested using real-time Quantitative Reverse Transcription PCR, in situ hybridization, microassays, and MPSS. The Real-Time Quantitative RT-PCR technology used in the study aimed to characterize gene expression patterns through quantification of messenger RNA (mRNA), by duplicating reverse transcription with PCR, as a substitute for cell metabolism (Sanders et al., 2014).

## 2. Methods

### *2.1 Materials and tools*

The materials used in the study of yoghurt beverages to see their function as immunomodulators, especially their effects on influenza, were different in the two studies. In the first study, the materials used were Madin-Darby canine kidney (MDCK) cells (Korean Cell Line Bank, Seoul, South Korea), mice lymphoma cell line, YAC-1 (Korea Cell Line Bank), 2 mM l-glutamine (Sigma-Aldrich), 25 mM HEPES (Sigma-Aldrich), 100 U/mL streptomycin, and 100 U/mL penicillin (Hyclone), yogurt (PY), whole-milk powder, and nF1 with heat treatment provided by Purmil Co. Ltd. (Seoul, South Korea), starter culture containing *Lactobacillus acidophilus, L. delbrueckii ssp. bulgaricus, S. thermophilus, Bifidobacterium, Lactobacillus paracasei* (*L. casei* 431), and *Bifidobacterium lactis* (BB-12) were obtained from Chr. Hansen A/S (Hørsholm, Denmark), heated nF1 powder with *L. plantarum* was purchased from Biogenics Korea Co. Ltd. (Seoul, South Korea), avertin (2,2,2-tribromoethanol and tert-amyl alcohol) was purchased from Sigma-Aldrich, and the IAV H1N1 strain (PR8) from A/Puerto Rico/8/34 mice that had been adapted was obtained from the International Vaccine Institute, Seoul, South Korea.

Meanwhile, in the second study, the material in the form of IAV H1N1 (PR8) strain from A/Puerto Rico/8/34 mice that had been adapted was maintained at the Kitasato Institute for Life Sciences, Kitasato University, Japan. The influenza HA vaccine was taken from the virus. Yogurt was fermented with *L. bulgaricus* OLL1073R-1 and *Streptococcus thermophilus* OLS3059 with the number of *L. bulgaricus* OLL1073R-1 and *S. thermophilus* OLS3059 cells in yogurt were 3.5×108 cfu/g and 6.8×108 cfu/g respectively. Other materials were 10% skim milk, 10% trichloroacetic acid (w/w), 0.05 M Tris–HCl buffer (pH 8.0) containing 1 mM $MgCl_2$, and treated with 2 μg/ml DNase (EC. 3.1.21.1; Sigma-Aldrich, St. Louis, MO, USA) and 2 μg/ml RNase (type I-AS, EC. 3.1.27.5; Sigma-Aldrich), and 0.2 mg/ml proteinase K (EC. 3.4.21.64; Sigma-Aldrich).

Experimental animals in the first study was 8-week-old male BALB/c mice purchased from Samtako (Osan, South Korea) while in the second study was 7-week-old female BALB/c mice purchased from CLEA Japan (Tokyo, Japan). The equipment used in the first study was a humidified incubator containing 5% $CO_2$ to maintain cells, 96-well cell culture plate, scale, biosafety level 2 unit, Qiagen RNeasy mini kit, RevertAid First strand cDNA synthesis kit (Thermo Fisher Scientific) with oligo-dTs, Rotor-Gene Q (Qiagen), cell strainer (pore size, 70 μm; SPL, Gyenonggi-do, South Korea), LDH assay kit, commercial LDH colorimeter kit, and microplate reader. The equipment needed in the second study was a biotinylation kit for the HA vaccine biotinylation process, anion-exchange chromatography with DEAE Sepharose Fast Flow for NPS and APS purification, intraperitoneal injection, FACSCalibur flow cytometer.

### *2.2 Experimental design*

Both researches studied the effect of yogurt as an immunomodulator, especially against influenza disease, using mice. The treatments in the two studies were different. In study 1, there were 4 treatments tested. The first treatment (PY1) was with yogurt fortified with nF1 in phosphate-buffered saline (PBS). The second treatment (PY2) was made by increasing the concentration of nF1 by 5 times (2.5 mg/d) compared to PY1 (0.5 mg/d). The milk treatment was in the form of powdered milk dissolved in PBS, and the PBS solution was used as the control group (CON). While in study 2, there were 3 treatments tested, namely yogurt fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1, EPS or Exopolysaccharides from the strain, water as a control, and further observations were made on NPS (neutral EPS) and APS (acid EPS).

The data obtained in study 1 were gene expression levels, cytokine levels, NK cell cytotoxicity, NK cell activity, spleen and thymus index, proinflammatory cytokine expression, and the protective effect of yogurt on mortality. In study 2, the data obtained were survival rates, NK cell activity, influenza antiviral Ab titer data, influenza virus infection data, and virus proliferation data. Data from study 1 were expressed as mean ± standard deviation while in the second study was expressed as mean ± standard error of the means (S.E.M.).

Significant differences compared to the control and treatment groups were analyzed using paired Student's t test, while in study 1, unpaired t test was also involved. If there was a significant difference, Tukey–Kramer test was performed. In study 2, differences in survival rates between groups after lethal influenza virus injection were analyzed using the log-rank test in the Kaplan–Meier method with the StatView® application (SAS Institute, Cary, NC USA). The difference was considered significant if the p-Value b was 0.05.

## 3. Results and Discussion

### *3.1 Principles and procedurs of analysis*

The principle of the test is when the antibody is mixed with the antigen, it will form an insoluble complex, which results in a change in the absorbance value on the spectrophotometer with a wavelength of 700 nm. The reading with the spectrophotometer can be compared with the known IgA concentration calibrator. The kit used in this analysis contains 2 reagents, namely 20 mL reagent buffer (R1) and reagent antiserum (R2). The test procedure begins with adding 250 µL of R1 into a test tube containing 5 µL of sample. Both are mixed, the absorbance measurement is carried out after 5 minutes. The measured absorbance value is then named A1. Furthermore, 70 µL of R2 is added to the test tube and the measurement is done.

#### *3.1.1 Specimen collection*

On the fourth day postinfection, blood was taken from the heart of dead mice with a syringe. Serum samples were separated from the blood by centrifugation. After that, the abdomen of the mice was incised along the median line from a small point at the end of the sternum to the tip of the chin. The trachea and lungs were removed and washed twice by injecting 2 ml of PBS containing 0.1% bovine serum albumin (BSA). The bronchoalveolar washing process was tested for antiviral Abs after cell debris was removed by centrifugation. Serum and BALF were stored at −80°C until used (Nagai et al., 2011). Meanwhile, in study 2, mice were euthanized. After that, their thymus and spleen were taken for further analysis. The spleen, thymus, and body weight were measured, and organ indices were calculated.

*3.1.2 Effect of treatment on influenza virus proliferation in the lungs of mice*

The infectious virus titer in BALF of mice infected with influenza virus was estimated on the fourth day after infection by measuring with plaque assay. Plaque assay is a bioassay method commonly used to evaluate antiviral compounds. Therefore, to avoid the volatilization of the test sample compounds, plaque assay was used to confirm the antiviral activity of the compounds in the test samples against influenza A/PR/8/34 H1N1 virus. The anti-influenza virus activity was tested using direct suppression of influenza virus replication in MDCK cells by plaque assay. MDCK cells grown in 6-well tissue culture plates were inoculated with 100 PFU/0.2 mL of influenza A/PR/8/34 H1N1 virus. After adsorption of the virus to the cells at room temperature for 30 min, the inoculum was removed. Cells were plated with 1.8 mL of nutrient agarose medium (0.8%) containing various concentrations of sesquiterpenes or zanamivir in 10% methanol solution (200 lL), and cultured in a humidified environment containing 5% $CO_2$ for 3 days at $37^0$C after solidification. Each plate was then coated with agarose staining medium containing 0.1% neutral red, and incubated at $37^0$C with 5% $CO_2$ for 180 min. The number of plaques was counted under a stereomicroscope. The effective concentration to reduce plaque by 50% (EC50) was determined from a curve showing the number of plaques and the concentration of the tested sample (Kiyohara et al., 2012).

3*.1.3 Effect of orally administered treatment on anti-viral influenza Ab titers*

On the fourth day after infection, yogurt and EPS were shown to increase the titer of anti-influenza virus IgA and $IgG_1$ Ab in bronchoalveolar lavage fluid (BALF) compared to only consuming water as a control. According to the source, subcutaneous injection of influenza vaccine is known to induce the production of serum antiviral IgG antibodies (Abs) which provide a protective effect against influenza virus proliferation in lung tissue. In addition to IgG, the vaccine can stimulate the production of mucosal IgA Abs which can fight influenza viruses when administered intranasally.

The procedure used in conducting the test was to use cuvettes on a 96-well ELISA plate coated with 100 µl of anti-mouse IgA or IgG1 mAb in 50 mM carbonate–bicarbonate buffer (pH 9.5) containing BSA (10 µg/ml), and incubated at 37°C for 3 hours. After the liquid was separated, the blocking solution, 1% nonfat dry milk in phosphate buffered saline (PBS), was placed in each curve (300 µl) and incubated at 37°C for 1 hour. The plate then washed for 3 times with PBS containing 0.05% Tween 20 (PBS-Tween). BALF or serum diluted for several series with SuperBlock® blocking buffer in PBS (Thermo Fisher Scientific) (diluted to 1:10 with PBS containing 0.05% Tween 20) was added to the curves (100 µl). The curves were then covered with adhesive tape and the plates were incubated overnight at room temperature. After rinsing with PBS-Tween, 1 µg/ml biotinylated influenza HA vaccine in blocking solution was added to each curve (100 µl). The plates were then incubated at room temperature for 1 h while stirred on a microplate mixer. After the curves were washed, streptavidin-β-galactosidase conjugate was diluted to 1:1000 with blocking solution, added, and incubated at room temperature for 1 h with stirring.

After the last wash, 0.1 mM 4-methylumbelliferyl-β-D-galactoside in buffer A (10 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 1 mM MgCl2 and 0.1% BSA) was added to each curve (100 µl). The curve plate was then covered with tape and incubated at 37°C for 2 h. The enzyme reaction would be stopped after the addition of 100 µl of 0.1 M glycine–NaOH (pH 10.3), and the fluorescence of 4-methylumbelliferone was measured (ex. 355 nm, em. 460 nm) using a Fluorescent II spectrophotometer. The endpoint of the specific antibody titer for influenza HA vaccine was expressed as the reciprocal log2 titer (Nagai et al., 2010).

### *3.1.4 Effect of orally administered treatment on NK cell activity*

There are differences in the method of testing NK cell activity in studies conducted by Nagai et al. (2011) and Kim DaHyun et al. (2018). In study 1 or by Kim DaHyun et al. (2018) NK cell activity from splenocytes was measured as NK cell cytotoxicity against target cells using the lactate dehydrogenase (LDH) test (Yu et al., 2017). To test NK cellactivity and inflammatory responses in infected mice, mice (n = 6 per group) were infected with a sublethal dose of PR8. On the fifth day after post-infection, the organs were collected for further analysis.

To obtain splenocytes, spleens were homogenized in Hanks' balanced salt solution and filtered using a cell strainer (pore size, 70 μm) immediately. Red blood cells were obtained using ACK buffer (150 mM $NH_4Cl$, 10 mM $KHCO_3$, and 0.1 mM $Na_2EDTA$) and washed twice with Hanks' balanced salt solution. Splenocytes were resuspended in RPMI 1640 (without phenol red) with 10% FBS, 100 U/mL streptomycin, and 100 U/mL penicillin (Hyclone). To measure the cytotoxicity of NK cells, YAC-1 was used as the target cell, while splenocytes were used as the effector cells. NK cell cytotoxicity against YAC-1 cells was measured by observing LDH leakage after 4 hours (effector:target = 50:1) using an LDH assay kit, and absorbance was measured at 490 nm using a commercial colorimetric LDH kit and a microplate reader. The percentage of NK cell activity was calculated by the formula: NK cell cytotoxicity (%) = (Ac - At) / (Al - At), Ac is the absorbance of the coculture medium, At is the absorbance of the target cell culture medium, and Al is the absorbance of the target cells; cell lysis buffer was provided in the LDH assay kit (Kim DaHyun et al., 2018).

Meanwhile, in a study by Nagai et al. (2011) the activity of NK cells from its splenocytes was assessed using flow cytometry (YAC-1 cells were used as target cells and $0.5 \times 10^6$YAC-1 cells/mL were labeled with 2.5 μg/mL of 3,3′-dioctadecyloxacarbocyanine perchlorate after overnight incubation at 37°C. The cells were then rinsed 3 times with RPMI 1640 medium and resuspended at the concentration of $2.5 \times 10^5$ cells/mL. Mice splenic lymphocyte effector cells ($10^6$/cuvette) were added to the target cells at $2.5 \times 10^4$ cells/cuvette (ratio 40:1) with a total volume of 200 μL/cuvette in a 96-well, round-bottomed plate. The samples were then centrifuged (30 × g, 1 min), and incubated for 4 h at 37°C in a humidified 5% $CO_2$-air atmosphere. Fifteen minutes before the end of incubation, 20 μL of propidium iodide (0.5 mg/mL in PBS; Sigma) was added to each cuvette to label dead cells. The extent of target cell lysis was determined using FACSCalibur flow cytometry, and NK cell activity was expressed as the percentage of their specific cell-lysing effectors (Nagai et al., 2011).

### *3.1.5 Quantitative reverse transcription PCR*

To test the levels of cytokine expression in the spleen of each sample group, total RNA was isolated from the spleen using RNAiso plus reagent and cDNA was synthesized using the LeGene Premium Express first-strand cDNA synthesis system. Gene expression levels were measured by quantitative reverse transcription PCR amplification using the iQ5 real-time PCR detection system with HiPi SYBR green 2× master mix. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) and normalization was performed using Rn18s as the housekeeping gene. To test the levels of cytokines in the lungs of infected mice, total RNA was extracted using Tri reagent and purified using the Qiagen RNeasy mini kit. cDNA synthesis was performed using the RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific) with oligo-dTs. Transcripts were quantified using primers for the target genes Il1b, Il6, and Actb. Real-time PCR was performed and analyzed on the Rotor-Gene Q (Kim DaHyun et al., 2018).

### *3.1.6 The effect of oral treatment on survival rates*

There were differences in observing the survival rate of mice in studies conducted by Nagai et al. (2011) and Kim et al. (2018). In the study by Kim DaHyun et al. (2018) to

evaluate the survival rate, mice were infected with PR8 at a dose of 2×LD50 (2×102.17 TCID50) per mice (n = 20 per group). Mortality was monitored until the 21st day post-infection. Mice that lost more than 25% of their initial body weight were injected following the protocol (No. KUIACUC-2016–61). Meanwhile, in the study by Nagai et al. (2011), the effect of fermented yogurt on the survival rate of mice infected with influenza virus was observed for 14 and 21 days.

### *3.2 Discussion of experimental methods and design*

#### *3.2.1 Effect of treatment on influenza virus proliferation in the lungs of mice*

Plaque assays are a standard method that has been used for long time to determine virus titer or infectious dose. This method can determine the number of plaque forming units (PFU) in a sample. Typically, a 10-fold dilution of the stock virus concentration is inoculated into each plate. Cells infected with the virus lyse and spread the infection to adjacent cells. As the cycle of infection and lysis repeats, the area of infected cells forms a visible plaque. The PFU/mL result indicates the number of infectious particles in the sample, assuming that each plaque is caused by a single infectious virus particle. It is important to note that plaque assays are limited to viruses that cause cell death or lysis, such as the influenza virus in this study, which forms plaques in cell culture plates. In fact, many viruses do not form plaques, but cause a visible cytopathic effect or CPE. The curves are examined for the presence of CPE over several days (Ryu, 2017).

Plaque assays are used to quantify discrete infection sites. A sample containing virus is serially diluted and an aliquot of each dilution is added to a plate of cultured cells. Cells are then infected by virions, leading to viral replication, producing progeny virions. Virions infect and kill cells around to produce holes or plaques that are visible to the eye (or under magnification). Often live cells are stained to provide a dark background for the clear plaques. The goal of making and testing serial dilutions is to achieve a countable number of plaques in a single layer of cells. The results are expressed as plaque forming units (PFU) per volume (Payne, 2017).

#### *3.2.2 Effect of orally administered treatment on anti-viral influenza ab titers*

On the fourth day after infection, yogurt and EPS could increase the titer of anti-influenza virus IgA and IgG1 Ab in BALF compared to only consuming water as a control when given orally to mice. Subcutaneous injection of this vaccine is known to induce the production of serum antiviral IgG antibodies (Abs) which provide a protective effect against influenza virus proliferation in lung tissue. An effective vaccine can be administered intranasally and stimulates the production of mucosal IgA Abs against influenza viruses. Studies using BALB/c mice showed that consumption of substances that have strong antiviral activity against H1N1 and H3N2 subtypes of influenza A and influenza B viruses can be evidenced by an increase in antiviral IgA Abs in nasal wash and bronchoalveolar lavage fluid (BALF).

The amount of IgA or IgG1 Abs for influenza virus in BALF was measured by a modified fluorometric reverse (Ab-capture) ELISA. The endpoint titer of influenza virus-specific Abs was expressed as the reciprocal $\log_2$ titer. It is known that anti-influenza virus Abs can protect the body from influenza virus infection. In the present study, yogurt fermented with and EPS produced by L. bulgaricus OLL1073R-1 increased anti-influenza virus IgA and IgG1 Abs in the BALF of mice infected with influenza virus. These results suggest that the addition of anti-influenza virus Ab may be associated with a reduction in the titer of infectious virus in the bronchoalveolar cavity of mice.

The presence of the mucosal immune system or common mucosal immune system (CMIS) and Peyer's patch cells which are one of the CMIS inductive sites in the gut-associated lymphoid tissue (GALT) can induce IgA Ab production. The results of a study using Lewis rats showed that lymphocytes from Peyer's patches successfully migrated to

the nasopharyngeal lymphoid tissue (NALT) and brought T cells containing many endothelia to the venules. This suggests the possibility that oral administration of EPS activated lymphocytes in Peyer's patches allowing them to migrate to the airway mucosa and increase the production of influenza virus-specific IgA Ab from antibody-secreting cells in the airway mucosa through CMIS (Nagai et al., 2010).

### *3.2.3 Effect of orally given treatment on NK cell activity*

Natural killer cell or NK cell activity is a commonly used parameter in determining the performance of lysis activity against tumors and virus-infected cells in innate immunity (Nishimura et al., 2017). The activity of NK cells from splenocytes measured by LDH assay is described as the cytotoxicity of NK cells against target cells (Yu et al., 2017). To confirm the immune-enhancing effect of nF1-enriched yogurt on IAV infection, NK cell activity from infected mice was examined after administration of yogurt for 21 days before infection. Splenic NK cells directly infected with the virus in vitro would lose their cytotoxicity, the expression of cytokines and chemokines decreases, and show a decrease in NK cell surface receptors. NK cells can prevent virus replication in infected cells by increasing IFN-γ production to eliminate infected cells (Vidal et al., 2011). In accordance with the upregulation of NK in activating cytokine gene expression, PY2 formula given to mice resulted in higher NK cell cytotoxicity than that seen in control group mice and milk group mice.

In addition, type 1 IFN and IL-12 are known to be cytokines that activate NK cells and alter their response to IAV infection. Interleukin-12 represents an NK-activating cytokine, and has been shown to enhance NK cell activity after infection and protect against IAV infection when administered prior to infection (Ishikawa et al., 2016). In addition, IFN-α released from macrophages increases NK cell production of IFN-γ and other cytokines when the body is infected with IAV. Thus, cytokines regulated by PY2 may reactivate NK cells prior to IAV infection and maintain high NK cell cytotoxicity. This is in accordance with previous studies on NK activity that increased BAL (LGG, *Lactobacillus pentosus* S-PT84 and *L. delbrueckii* OLL1073R-1) in lung or spleen tissue infected with influenza virus (Harata et al., 2010; Nagai et al., 2011; Makino et al., 2016).

LDH activity associated with NK cell-mediated target cell killing was measured by colorimetric, and the percent cytotoxicity was calculated using the existing formula. In the LDH release assay, LDH activity derived from lysed target cells in the media was measured as the absorbance after incubation of effector cells with target cells (Nishimura et al., 2017). According to the study (Hassenrück et al., 2018), LDH released from lysed cells caused the reduction of iodo tetrazolium chloride. The amount of colored formazan formed was measured by absorption at 450 nm in a plate reader. Effector cells were placed, followed by target cells and 10 μg/ml mAbs. Low and high controls, the state of LDH release either spontaneously or maximally, were determined from target cells alone or after lysis of target cells plus effectors fully-occurred by 1% Triton X-100.

Meanwhile, in measuring the activity of NK cells from mice splenocytes using the flow cytometry method, NK cell activity is expressed as the percentage of lysis of specific effector cells (Nagai et al., 2011). In this study, oral administration of yogurt and EPS increased NK cell activity in splenocytes from mice infected with influenza virus. In this case, yogurt fermented with and EPS produced by L. bulgaricus OLL1073R-1 could increase cellular immunity, and protect against influenza virus infection, because NK cells are lymphocytes from the innate immune system that play an important role in the initial defense against various viral infections. The mechanism that occurs in increasing NK cell activity was assumed to be due to EPS carried by Peyer's patches in the intestine stimulating antigen cells, such as dendritic cells, through receptors. This will result in a selective increase in the proliferation of T-helper 1 (Th1) cells, and continued production of IL-2 and IFN-γ, which are important cytokines for the immune response (Nagai et al., 2011).

Flow cytometry uses fluorescent dyes instead of radioisotopes to measure NK cell activity. This approach can distinguish targets from effectors by labeling targets with

fluorescent dyes, such as carboxyfluorescein diacetate succinimidyl ester (CFSE) and DiO, which have excitation and emission properties similar to fluorescein isothiocyanate (FITC). The percentage of total PI+ targets can be measured using flow cytometry because dead target cells can be distinguished from viable cells by staining with propidium iodide (PI) (Nishimura et al., 2017).

This method can also be used to study the regulatory capacity of NK cell migration as a result of genetic/epigenetic changes (up or down regulation) or arising from drug therapy. For all experiments using conditioned media, it is important to ensure equivalent conditioned media are used in control and treatment conditions to obtain accurate measurements (Chava et al., 2020).

*3.2.4 Quantitative reverse transcription PCR*

To evaluate the immune enhancing effects of the treatment, mice were given yogurt orally every day and their organs were analyzed using Quantitative Reverse Transcription PCR. This technology is used to characterize gene expression patterns through quantification of messenger RNA (mRNA), by amplifying reverse transcription by PCR, as a surrogate for cell metabolism. To test the effect of the treatment on cytokine expression, splenic cytokine mRNA transcript levels were measured. Gene expression is a marker of cytokines and NK cells in the spleen (Sanders et al., 2014).

In this study, Ifna, IL-2, IL-12, Klrb1, and Cd69 transcript levels were measured in the spleen by Quantitative Reverse Transcription PCR using Rn18s as a normalization control after 4 weeks. The study showed that nF1-enriched yogurt improved immune function in immunocompetent and immunosuppressed subjects. In accordance with previous studies, increased thymus and spleen weights could be used as markers of increased immunity, seen from increased weight along with immune response (Li et al., 2018 in Kim DaHyun et al., 2018; Sun et al., 2017). In addition, the relationship between high thymus index and protection from IAV in this study suggested that consuming yogurt has an effect on IAV infection (Wang et al., 2016).

Type I IFN, IL-2, and IL-12 are produced by various immune cells upon viral infection, and cytokines can activate NK cells through specific cytokine surface receptors (Marçais et al., 2013). Cytokine stimulation induces NK cells so that surface marker expression, such as CD25 and CD69, is increased in cytolytic NK cells (Wehner et al., 2011; van Ostaijen-Ten Dam et al., 2016). Activated NK cells have stronger cytolytic activity because they are more able to produce cytolytic effector molecules, such as perforin and granzymes, which can cause lysis of damaged cells (Kim DaHyun et al., 2018).

3*.2.5 Effect of treatment on mice survival rate*

The effect of yoghurt fermentation on the survival of mice infected with influenza virus was observed for 14 and 21 days. The survival rate of mice infected with a lethal dose of IAV further demonstrated the protective effect of yogurt consumption. Daily administration of yogurt might provide a prophylactic effect due to an increase in the basal immune response. Since the gut microbiota is also said to play an important role in the development and function of lymphoid cells, including NK cells, yogurt might induce immunological response that increases NK cell activity and exhibits protective and prophylactic effects against IAV infection by changing the composition of the gut microbiome, thereby affecting the survival of mice.

*3.2.6 The advantages and disadvantages of the method*

In the LDH method, in vitro protocol can reliably and reproducibly monitor the effects of cytotoxicity on cancer cells (or other target cells). This protocol can test without involving radioactivity, is easy to perform, sensitive, can be scaled up for higher throughput screening, and reproducibly identifies factors that modulate NK cell function. This method is designed

to be adaptable to most laboratories with direct colorimetric, microscopic, or FACS-based readouts that are easy to interpret and allow researchers to reach precise conclusions.

However, this method has a drawback, namely the inability to see changes in NK cell migration. However, flow cytometry can be a solution to this problem because this method is a quantitative method and can monitor NK cell migration. Previously, the effector function of NK cells could mostly only be monitored by degranulation and cytotoxicity tests as well as chromium release which required several specific prerequisites, including the need for a gamma counter, which based on radioactive that requires training in its use. The disposal of radioactive materials are also risky for its users (Chava et al., 2020).

Reverse transcription quantitative PCR is a widely accepted and applied method. This method is also relatively easy, and effective for RNA measurement. Reverse transcription qPCR (RT-qPCR) allows rapid and precise assessment of changes in gene expression as a result of physiology, pathophysiology or development (Sanders et al., 2014).

## 4. Conclusions

Yogurt has immunomodulatory properties that if consumed regularly can affect the immune system and protect against IAV infection with the presence of extracellular polysaccharides, as presented in study 2. Daily consumption of yogurt before infection also increased NK cell activity, cytokine response in the lungs, and the survival rate of mice. The content in yogurt has been shown to induce IFN-γ production in mice splenocytes. Yogurt fortified with nF1 could induce immunological responses by increasing NK cell activity and also shows protective and prophylactic effects against IAV infection as a result of its ability to change the composition of the gut microbiota.

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