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Effect of Tempeh Supplementation on the Profiles of Human Intestinal Immune System and Gut Microbiota

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Tempeh is a traditional fermented soybean product from Indonesia. Although tempeh is consumed as daily menu in Indonesia, its nutrigenomic study employing human has not been reported yet. On the other hand, our study in mice showed that tempeh could enhance immune system, especially by increasing secretory immunoglobulin A production in ileum and colon. Tempeh was also found to be potential in modulating composition of gut microbiota. Therefore, the objective of this study was to analyze the impact of tempeh supplementation on the profiles of human intestinal immune system and gut microbiota analysis. A total of 16 participants, comprising of each 8 healthy females and males, aged between 20 and 23 were recruited to this study. The volunteers consumed 200 mL milk from day 1-8 followed by consumption of 100 grams steamed tempeh each day from day 9-24. Fecal samples taken on day 9 and 25, were analyzed with half sandwich ELISA for IgA enumeration. In addition, fecal samples collected on day 0, 9, and 25, were analyzed for total *Akkermansia muciniphila* employing quantitative real time PCR. The result of this study suggested that tempeh supplementation might act as paraprobiotic and slimming agents since tempeh enhanced production of IgA and increased population of *A. muciniphila* in human intestinal tracts.

Key words: *Akkermansia muciniphila*, ELISA, IgA, RT-PCR, tempeh

Tempe merupakan produk fermentasi kedelai asal Indonesia. Meskipun tempe dikonsumsi sebagai menu sehari-hari di Indonesia, studi nutrigenomik tempe dengan subjek penelitian manusia belum pernah dilaporkan sebelumnya. Studi nutrigenomik tempe dengan tikus sebagai hewan model menunjukkan bahwa tempe dapat meningkatkan sistem imun, yaitu dengan meningkatkan produksi immunoglobulin A sekretori di usus halus dan usus besar. Tempe juga memiliki potensi dalam memodulasi komposisi mikrobiota usus. Tujuan dari penelitian ini yaitu menganalisa efek suplementasi tempe terhadap sistem imun usus manusia dan menganalisa perubahan komposisi mikrobiota usus. Sebanyak 16 responden, yang terdiri dari 8 wanita dan 8 pria sehat, berumur 20-23 tahun berpartisipasi dalam penelitian ini. Responden mengkonsumsi 200 mL susu dari hari ke 1-8, dan 100 gram tempe dari hari ke 9-24 setiap harinya. Sampel feses diambil pada hari ke 9 dan ke 25 untuk perhitungan IgA dengan ELISA, sedangkan sempel feses yang diambil pada hari ke 0, 9, dan 25 dianalisis untuk perhitungan *Akkermansia muciniphila* dengan *quantitative real time PCR*. Hasil penelitian ini menunjukkan bahwa suplementasi tempe dapat berperan sebagai agen paraprobiotik dan penurun berat badan karena tempe mampu meningkatkan produksi IgA dan meningkatkan jumlah *A. muciniphila* dalam saluran pencernaan manusia.

Kata kunci: *Akkermansia muciniphila*, ELISA, IgA, RT-PCR, tempe

Tempeh is a traditional fermented soybean product from Indonesia. *Badan Standarisasi Indonesia* (BSN) also known as Standardization Body of Indonesia (2012) reported that 81 000 tempeh producers produce 2.4 tons tempeh every year in Indonesia. Tempeh is formed as the result of microorganisms' work, such as *Rhizopus* spp. and lactic acid bacteria. As the result of fermentation product, tempeh became easily digested. Since tempeh also has higher amino acids compared to raw soybean, tempeh could play an important role in intestinal cells proliferation and affect gut microbiota composition. Previous study had shown that tempeh has beneficial effects for immune system and cardiovascular health (Babu *et al.* 2009).

Tempeh nutrigenomics studies were mostly conducted in mice and rats as animal models and have not been reported yet to involve human as research object (Utama *et al*. 2013). Previous study showed positive impact of tempeh consumption, that tempeh supplementation increased ileum immunoglobulin A (IgA) gene and protein expressions in Sprague Dawley Rats (Soka *et al*. 2015 and Soka *et al*. 2015). Furthermore, Nurrahman *et al*. (2011) reported that tempeh consumption in *Salmonella typhimurium*-induced rats had enhancement of IgA concentration

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and accelerated the rats' resilience.

Immune system exploration is commonly detected by analyzing gut microbiota in the host's digestive system. Human gut microbiota contains at least 10^{11} bacterial cells per mL intestinal fluid. This microorganism's consortium could act as the source of energy and nutrition for intestinal metabolism. Intestinal stability is also guaranteed by the presence of probiotics, which is enhanced by the presence of prebiotics (Flint *et al*. 2010). In this study, tempeh's ability as prebiotic agent will be tested and one of probiotics bacteria, such as *Akkermansia muciniphila* would be enumerated.

Therefore, the objective of this study was to analyze the impact of tempeh supplementation on human's immune profiles, specifically by enumerating IgA level and *A. muciniphila* in fecal samples.

MATERIALS AND METHODS

Human Study. This study was reviewed and approved by the ethics committee of Research and Community Service Center in Atma Jaya Catholic University of Indonesia for employing human as research subject. Research was done by following all institutional guidelines.

A total of 8 healthy females and 8 healthy males aged 20-23 years volunteered for this study. Woman volunteers were neither pregnant nor breast-feeding. The volunteers were not smoking, did not consume any antibiotics at least one month before the intervention, and did not suffer lactose intolerance. The volunteers were required to be discipline in consuming given tempeh regularly and willing not to consume any kinds of prebiotics and other tempeh during the intervention.

The intervention was designed for 24 days. At day 0-8, the volunteers consumed 200 mL ultra-heat temperature (UHT) milk per day. At day 9-24, the volunteers consumed 100 grams of 10-minutes-steamed tempeh EMP. Fecal specimen collection was done in day 0, 9, and 25. Fecal specimen for IgA analysis was stored in -80 °C and fecal specimens for gut microbiota analysis were stored in -20 °C for further analysis.

Fecal Immunoglobulin A Extraction. The method was adapted from Peters *et al*. (2004). One gram of fecal specimen was diluted in 10 mL extraction buffer containing 0.01 M phosphate buffer saline (PBS) pH 7.4 containing 0.5% w/v Tween-20, and 0.05% w/v sodium azide. The fecal specimen was homogenized by mechanical homogenization with a vortex mixer. The fecal suspension was centrifuged at 1500 × g for 20 min at 5 °C. Two milliliters of supernatant was transferred to sterile tubes containing 20 µL protease inhibitor cocktail (Sigma-Aldrich, St Louis, Missouri, USA), which were prepared by following the manufacturer's instruction. The mixture was homogenized using vortex mixer and centrifuged at 10000 × g for 10 min. The supernatant was transferred to sterile tubes and stored in -20 °C.

Immunoglobulin A Analysis. Ninety-six well polystyrene plates (Nunc Maxisorp®, Wiesbaden, Germany) were coated with human colostrum IgA (Sigma-Aldirch, St. Louis, MO, USA) and fecal IgA samples, diluted in PBS (1:10) and incubated for 18 h at 4 °C. The next day, the plates were washed with PBS Tween for 4 times. The remaining protein sites were blocked with blotto, consisting of 5% skim milk in PBS Tween for 1 h at 37 °C. The plates were washed then added with rabbit anti-human IgA labeled with horseradish peroxidase (Sigma-Aldirch, St. Louis, MO, USA) diluted in blotto (1:10000) and incubated for 1 h at 37 °C. The plates were washed and added with 3,3',5,5'-tetramethylbenzidine as a substrate diluted in phosphate citrate buffer. The colorimetric reaction was stopped by adding 2 N sulfuric acid. The absorbance was measured with ELISA reader (Bio-Rad, San Fransisco, CA, USA) at 450 nm. Data were standardized into the IgA standard curve, and IgA concentration was expressed in ng mL^{-1} fecal samples.

Total Fecal Bacterial DNA Extraction. Bacterial DNA quantification method was adapted from Soka *et al*. (2014). The extraction was done using QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany) by following the manufacture's instruction. The modification was done by adding glass bead in sample homogenization step.

Standard Curve Construction and Bacterial Specific Enumeration. pGEM®T plasmid containing 16s rDNA of *A. muciniphila* was obtained from PT Nutrifood Indonesia (Jakarta, Indonesia) which was adapted from from Prawira (2014). The plasmids were transformed by employing *Escherichia coli* TOP 10 using $CaCl_2$ and $MgCl_2$ method. Plasmid extraction was done using Wizard® Plus SV Minipreps DNA Purification System (Promega, San Luis Obispo, CA, USA) by following the manufacture's instruction. Plasmid concentration serially diluted for DNA template in standard curve construction with a minimum five standard concentration between 10^4-10^{10} DNA copies per reaction. PCR primers and amplification for *A. muciniphila* was conducted and reported previously (Derrien 2007).

A. muciniphila from bacterial DNA and diluted plasmid were quantified with iQ5 Multicolor Real Time PCR Detection System (Bio-Rad, San Fransisco, CA, USA). A reaction was consisted of 10 µL KAPA SYBR® Fast Master Mix 2X Bio-Rad iCycler ™, 1 µL 10 ρmol μL^{-1} of each specific primers, 1 µL DNA template (100 ng mL^{-1}), and 7 µL nuclease free water, with total volume 20 µL. Condition applied for this analysis consisted of 1 cycle of 94 °C for 5 min and 40 cycles of 94 °C for 20 s, 50 °C for 20 s, and 72 °C for 50 s. Every diluted plasmid samples fecal DNA samples were duplicated with the same PCR reaction.

Statistical Analysis. Significant difference between before and after intervention were analyzed using paired parametric T-Test ($p<0.05$). Data was analyzed using Graphpad Prism version 6.0 (Graphpad Software Inc, La Jolla, CA, USA). Presented data is the mean of personal data from each volunteer ± standard error mean (SEM).

RESULTS

From a total of 8 female and 8 male participants, 3 male and 3 female participants were excluded due to their low physical fitness and less fulfillment of tempeh consumption during the intervention.

Fecal samples were stored for 6 weeks in -80 °C before IgA extraction process. The data shows that both male and female shows enhancement of IgA production (Fig 1) and the enhancement of *A. muciniphila* population in fecal samples (Fig 2). Higher secretion of male IgA was seen bigger in day 25 compared to day 9, i.e. 2573 ng mL^{-1} vs 2098 ng mL^{-1}, respectively, while in female i.e. 2421 ng mL^{-1} vs 2376 ng mL^{-1}, respectively. When the data is combined between male and female, the same pattern also occurred that higher IgA secretion was seen on day 25 compare to day 9, i.e. 2497 ng mL^{-1} vs 2237 ng mL^{-1}.

The number of *A. muciniphila* also increased after day 9 and day 25 compared to the beginning of experiment, day 0. From sampling on day 0, 9, and 25, male IgA enumeration was the highest after tempeh intervention on day 25, while on female the highest number of *A. muciniphila* was on day 9, before tempeh intervention. If male and female data were combined, the result showed the enhancement of *A. muciniphila* population kept increasing and was the highest after tempeh supplementation on day 25.

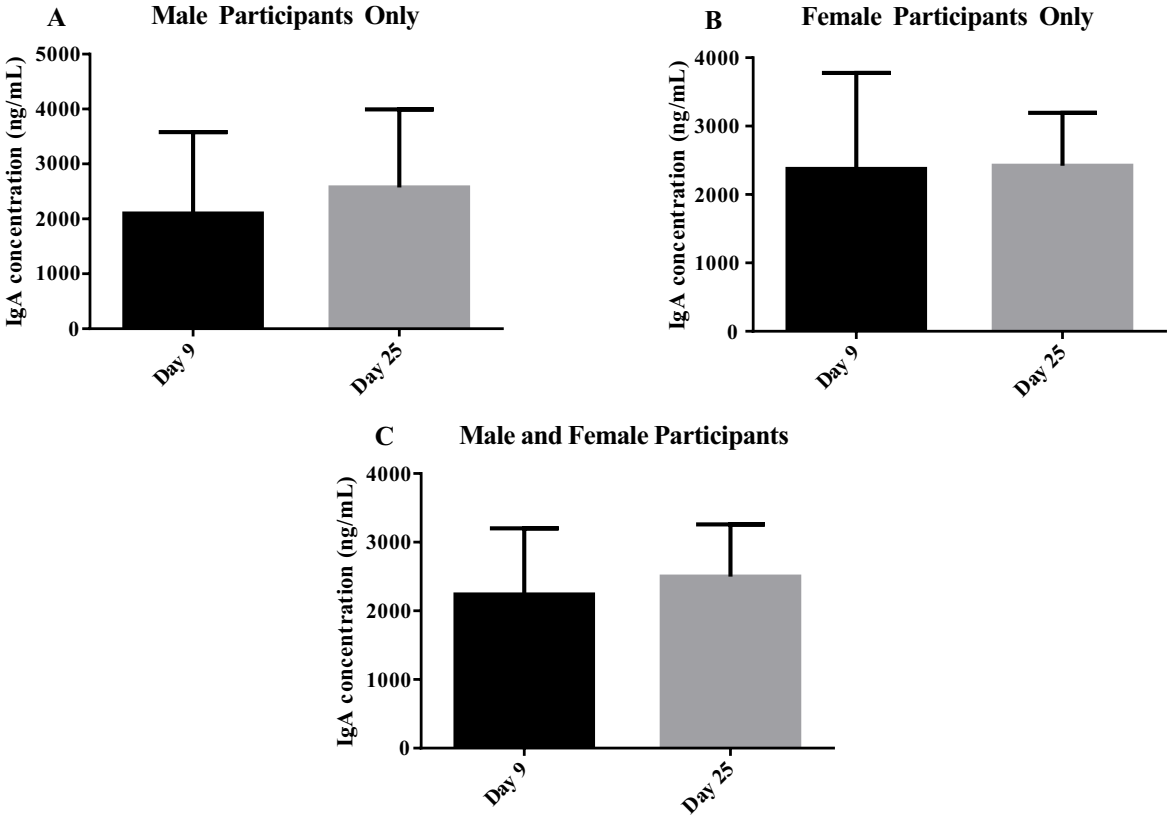

Fig 1 IgA level in fecal samples in male participants only (A), female participants only (B), and the combination between male and female participants (C). Day 9 represents the day after milk intervention and day 25 represents the day after tempeh intervention. Values are in ng mL^{-1} fecal IgA samples ±SEM (n=5 for each male and female).

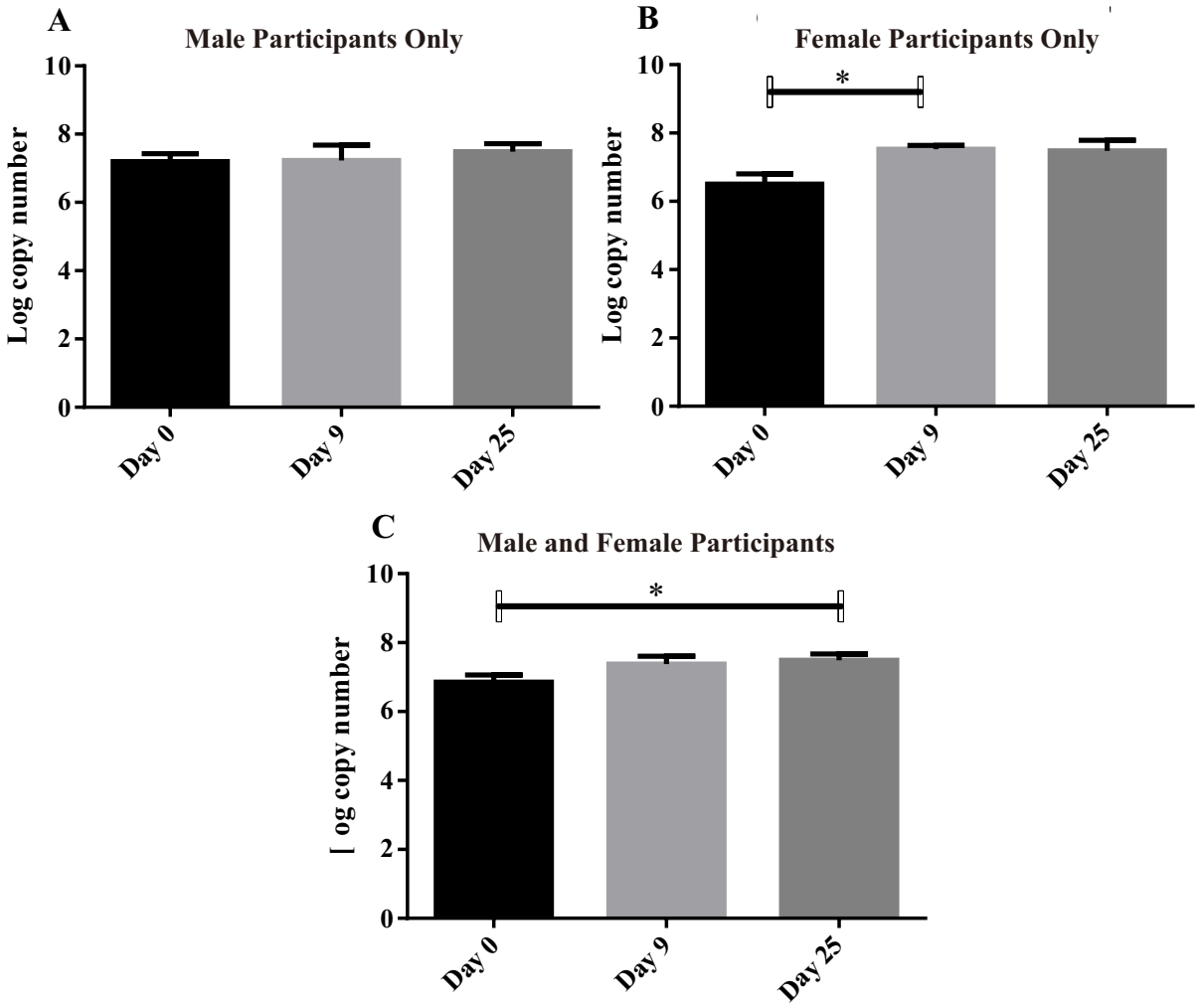

Fig 2 *Akkermansia muciniphila* level in fecal samples in male participants only (A), female participants only (B), and the combination between male and female participants (C). Day 0 represents the day before any intervention, day 9 represents the day after milk intervention, and day 25 represents the day after tempeh intervention. Values are in log copy number of 16s rDNA gene in fecal samples ±SEM (n=5 for each male and female). One asterisk represents significant difference between two days of intervention.

DISCUSSION

All participants were uniformed with UHT milk consumption. In general, UHT milk, which was taken from cow, and tempeh, which was made from soybean contain different protein profile. These facts brought the expectation of discrete results on before and after tempeh intervention.

On the other hand, tempeh is rich of various microorganisms that form a distinctive flavor of tempeh. The variations may occur due to differences of tempeh raw material and procession steps from the producers. Tempeh empang (EMP), which is made in Empang, Bogor was used in this study as it was previously explored before (Soka *et al*. 2014, Soka *et al*. 2015). EMP is produced by 6 main steps, which are soybean cooking, soaking, de-hulling, washing, mixing with inoculum, and incubation until tempeh was ready for harvest in 2-3 days. Metagenomics study showed that EMP tempeh had higher number of bacterial cells, compared to WJB tempeh, which was produced in Warung Jambu, Bogor. EMP provides more antigens compare to WJB tempeh. *Acetobacter indonesiensis*, *Klebsiella pneumoniae*, *Bacillus subtilis*, and *Flavobacterium* sp. were dominant bacteria found in EMP tempeh, while *Klebsiella* sp. and *Pseudomonas putida* were the dominant bacteria in WJB tempeh (Barus *et al*. 2008). A study by Ayu *et al*. (2014) showed that *Klebsiella pneumoniae* from Indonesian tempeh were genetically different from pathogenic isolates. A study by Soka *et al*. (2014) suggested that Indonesian tempeh might modulate the composition of gut microbiota toward a healthier gut. In addition, Soka *et al*. (2015) also reported that Indonesian raw and cooked tempeh might stimulate IgA secretion, and also both viable and non-viable

microorganisms might be stimulating IgA gene expression in Sprague Dawley Rats.

Secretory immunoglobulin A (sIgA) is an antibody which presence in mammals' digestive tract, including human. sIgA act as a first defense barrier for protecting the intestine from pathogenic bacteria and toxins. sIgA production against specific antigens is likely influenced by the presence of antigen-presenting cells, such as dendritic cells. The performance of this antibody is influenced by the activation of T cells and B cells (Mantis *et al*. 2011). sIgA also has an important role in the activation of various non-inflammatory pathways and control intestinal microbiota balance (Corthesy 2010).

The production of sIgA is affected by the presence of antigens in the intestinal tract. sIgA is the form of two dimeric IgA that is connected thru J Chain. This dimeric structure prevents sIgA from proteolytic enzyme activity, so then sIgA works optimally to protect mucosal immune system (Woof and Russel 2011). In this study, monomeric IgA level was measured. IgA would not present in membrane-bound form and would not be attached to J-chain. Therefore, IgA should be easier to be quantified to see the reflection on intestinal immune profile.

Since EMP tempeh was previously steamed for 10 minutes, the microorganisms in tempeh became inactive in our bodies. Although the microorganisms were made to be inactive, intestinal immune cells could recognize the dead cells as antigens and lead to IgA stimulation in the form of intestinal immune system defense. This concept is also known as paraprobiotics effect (Taverniti and Guglielmetti 2011).

Milk supplementation was done as comparison to tempeh supplementation. For the first 8 days, the volunteers consumed 200 mL milk, which could act as prebiotic agent due to the presence of galactooligosaccharides (Patel and Goyal 2012). Since the volunteers were not allowed to consume any probiotics during the intervention, it might lead to IgA reduction. IgA production is affected by the presence of probiotics bacteria (Anandharaj *et al*. 2014). In our findings, milk supplementation could increase probiotic bacteria and led to IgA production, which might explain that IgA concentration on day 9, as the baseline was pretty high and close to IgA concentration after tempeh intervention.

Tempeh intervention was done for the next 16 days and the concept of paraprobiotics was proven. Our findings showed that tempeh intervention slightly increased IgA production compared to milk intervention on day 9. The result also showed that tempeh could act as prebiotics and paraprobiotics agents for human body because tempeh contains reducing sugars and significant amount of dead cells.

Akkermansia muciniphila is a gram negative bacteria belonging to *Verrumicrobia* phylum. *A. muciniphila* colonizes in cecum, mucin's biggest production site in digestive tract. This bacterium has the ability to degrade mucin, a glycoprotein which composed of serine and threonine peptides and linked by O- and N- glycosidic bond. Mucin has four main oligosaccharides, which are N-acetyl glucosamine, N-acetyl galactosamine, galactose, and fucose. Mucin degradation employs several enzymes, such as glycosidase and sulphatase that disrupt oligo-saccharide bonds. The presence of mucin provides advantage for *A. muciniphila*, which acted as alternative energy source for intestinal metabolism. *A. muciniphila* regulates immune system, cell proliferation, cell adhesion, and cell apoptosis and mucosal gene expression (Derrien *et al*. 2004; Derrien *et al*. 2011).

Previous study also reported that *A. muciniphila* dominates 3-5% gut microbial communities of healthy people. *A. muciniphila* also shows to have positive correlation with obesity and type 2 diabetes reductions since endocanabinoid component controls inflammation, intestinal balance, and intestinal peptides secretion. Moreover, the presence of *A. muciniphila* also increases 2-oleoglyserol level, which stimulates the secretion of glucagon-like-peptide from L cells (Everard *et al*. 2012).

Both milk and tempeh were tested to act as prebiotic agents since milk contains galactooligo-saccharides and tempeh contains various peptides and oligosaccharides. The result showed that tempeh supplementation increased the number of *A. muciniphila* significantly on combined male and female participants, compared to day 0, or before intervention. The increase of *A. muciniphila* population in this study suggested that consuming tempeh could be promising for weight loss and to reduce diabetes type-2 syndrome.

To conclude, our findings suggested that tempeh supplementation might modulate human intestinal immune system by increasing IgA production. In addition, tempeh consumption could increase population of *A. muciniphila*.

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