Bioactivity of Soybean Tempeh against Diarrhea Associated Pathogen Is More Correlated with the Number of Total Bacteria than Specific Major Bacterial Phylum

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Abstract: Soybean tempeh can reduce the severity of diarrhoea through the inhibition of enterotoxigenic Escherichia coli (ETEC) adhesion to intestinal cells. This bioactivity is due to the presence of bioactive oligosaccharides derived from degradation of soybean matrix by fungi. Tempeh also contains other microorganisms such as bacteria and there has been no report whether bacteria can also influence the anti-adhesion bioactivity of tempeh extract against ETEC. In this research, we quantified bacterial population in tempeh samples using real time polymerase chain reaction (RT-PCR) method and measured the anti-adhesion bioactivity against ETEC of the extract using yeast agglutination assay. Data from both analyses were compared to see if there is any correlation between the two variables. Bacterial quantification with RT-PCR was focused on the enumeration of total bacteria and two specific major bacterial phyla in tempeh: Firmicutes and γ-Proteobacteria. There was a significantly strong positive correlation (R = 0.733) between total number of bacteria with anti-adhesion bioactivity of tempeh. However, there was no strong correlation between the number of Firmicutes and γ-Proteobacteria with anti-adhesion bioactivity. Our finding indicates that the anti-adhesion bioactivity of tempeh tends to increase following the abundance of bacteria but is not significantly affected by specific major bacterial phylum.

1 INTRODUCTION

Tempeh is a traditional Indonesian food made from the fermentation of legumes, most commonly soybeans, by the mold *Rhizopus oligosporus* (Nout & Kiers, 2005). At the end of fermentation process, fungal mycelia will bind the soybeans together resulting in a firm and compact cake. The firm texture of the end product is due to the activity of various fungal enzymes that break down the soy matrix thus increasing the digestibility and nutritional value of the substrate (Nout & Kiers, 2005). One of the products derived from the fungal enzymatic activity during fermentation is bioactive oligosaccharides that have been reported to have anti-diarrheal bioactivity (Kiers et al., 2002). This bioactivity is due to the capability of bioactive oligosaccharides in binding with the fimbriae of enterotoxigenic *Escherichia coli* (ETEC) thus inhibiting adhesion of the pathogenic bacteria to intestinal cells (Roubos-van den Hil et al., 2010). This results in the lower incidence of diarrhea since the pathogenicity of ETEC is determined by its capability of adhering to the intestines to produce enterotoxins (Nataro & Kaper, 1998).

Other microorganisms other than fungi, such as bacteria, are also present in tempeh (Seumahu et al., 2013). Bacteria in particular plays an important role in tempeh production for the acidification of soybeans to inhibit the growth of spoilage microorganism (Nurdini et al., 2015). Characteristics and nutritional content of tempeh can also be influenced by bacteria such as bitterness in tempeh that is correlated with

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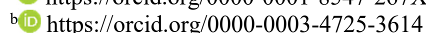
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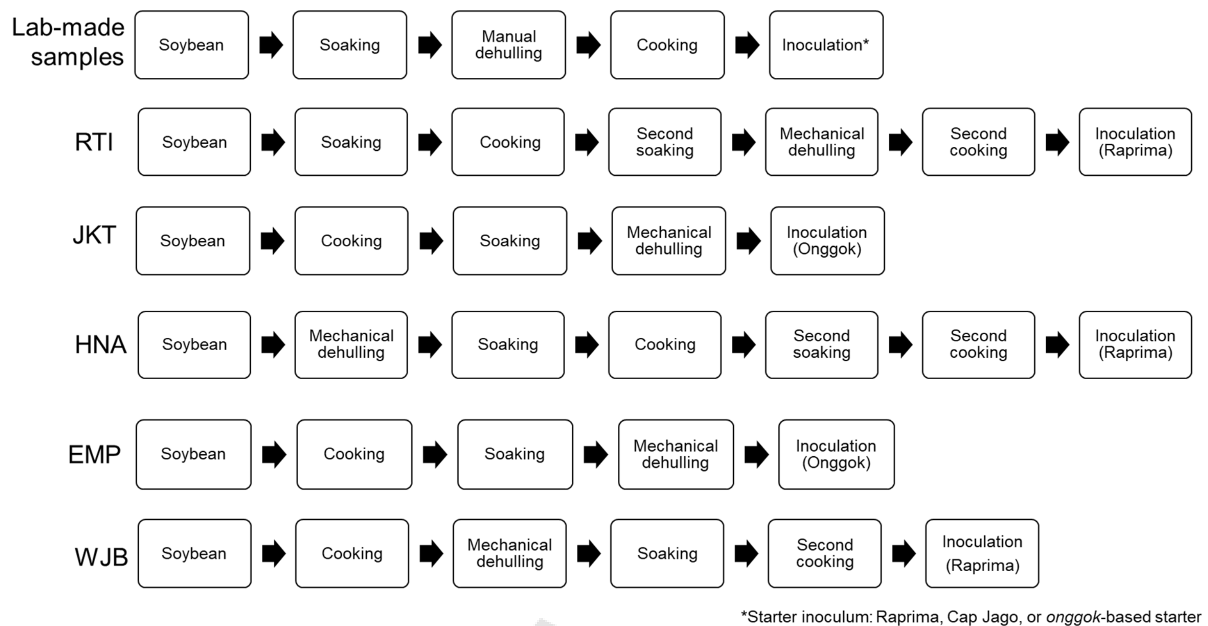

Figure 1: Major steps involved in the production process of the samples used in this experiment.

proteolytic bacteria and vitamin B12 in the product that is produced by bacteria from the genus of *Klebsiella* (Keuth & Bisping, 1994). Considering the abundance of bacteria in tempeh, a question arises whether or not these bacteria can influence the anti-diarrheal potential in tempeh.

The bacterial population in tempeh is dominated by the phylum Firmicutes (Radita et al., 2018). Roubos-van den Hil et al. (2010) demonstrated that fermentation of soybeans by *Bacillus* sp. can also result in anti-adhesion bioactivity against ETEC but the same activity was not observed in soybeans inoculate with *Lactobacillus* sp.. However, most reported experiments so far are focused on the fermentation of soybean by single bacterial or fungal culture. It is possible that in real life tempeh fermentation, these microorganisms might support or hinder one another during the breakdown of soy matrix polysaccharides thus influencing the level of anti-adhesion bioactivity. For example, bacteria could produce a polysaccharide-degrading enzyme allowing the substrate to be more accessible for further degradation by fungi. The opposite could also take place, that bacteria might consume the bioactive oligosaccharides thus decreasing bioactivity.

In this research, we focused on the correlation between anti-adhesion bioactivity against ETEC from tempeh extract with the population of all bacteria and two specific phyla: Firmicutes and γ-Proteobacteria. We decided to focus on those two phyla because both are reported to be the two major phyla found in tempeh (Radita et al., 2017). Firmicutes in particular can produce bacterial exopolysaccharides (EPS) that can bind to ETEC fimbriae (Wang et al., 2010). We measured the anti-adhesion bioactivity of tempeh extract using yeast agglutination assay, which *Saccharomyces cerevisiae* act as a model organism for eukaryotic cells. Real-time polymerase chain reaction (RT-PCR) was used as a method for bacterial quantification. Data from both experiments were statistically analyzed to determine the possibility of correlation between anti-adhesion bioactivity with the abundance of all bacteria or certain specific phylum.

2 MATERIALS AND METHODS

2.1 Materials

Tempeh samples used in this experiment consisted of five commercial tempeh and three tempeh made in laboratory condition with a variation of starter culture. All tempeh samples were made from the same type of yellow-seeded soybeans. Commercial samples were purchased from tempeh producers in Bogor (EMP, WJB, and RTI), Jakarta (JKT) and Surabaya (HNA). EMP, WJB, and JKT were produced by home-scale industries with the uncontrolled condition during the production process while RTI and HNA were produced by standardized industry with proper environmental control. Tempeh samples made in the laboratory were prepared with three different starters: the commercial starters

Raprima (RP; PT. Aneka Fermentasi Industri, Bandung, Indonesia), Cap Jago (JG; UD. Jaya Mulya, Kediri, Indonesia) and cassava-based *onggok* starter (OG; acquired from a traditional producer in Cisauk, Banten, Indonesia). Full-fat yellow-seeded elongated soybeans for tempeh fermentation were purchased from Pasar Modern Intermoda BSD (Tangerang, West Java). All tempeh samples were transported and kept at 4ºC prior to analysis. Figure 1 details the production steps of each sample.

Standard curves for RT-PCR were generated using pure cultures of *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella enterica* ATCC 51741 grown overnight in Luria broth at 37 ºC. *S. cerevisiae* and ETEC cultures were used for yeast agglutination assay and obtained from the Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia (Cisauk, Indonesia). D-(+)-mannose (Sigma Aldrich, Darmstadt, Germany) was used as a positive control for yeast agglutination assay.

2.2 Tempeh Fermentation

Lab-made tempeh with starter culture variation were prepared in the Laboratory of Microbiology, Atma Jaya Catholic University of Indonesia following the protocol detailed by Nout and Kiers (2005) with modifications. Full-fat yellow-seeded elongated soybeans were soaked overnight in distilled water at room temperature. Following overnight soaking, the soybeans were boiled and dehulled. The soybeans were cooled, dried, and divided to three experimental groups, each group weighing the same amount. Each group was mixed thoroughly with a tempeh starter (0.2 % (w/w) of soybeans) and packed in a perforated plastic bag followed with incubated at 30 ºC for 48 h. The final products were immediately used for the bioactive oligosaccharide and bacterial DNA extraction.

2.3 Extraction of Bioactive Oligosaccharides

Extraction of bioactive oligosaccharides from tempeh was based on the method described by Roubos-van den Hil et al. (2010) with modifications. Tempeh samples were lyophilized for 96 h and homogenized to obtain tempeh powder. About 1 L of distilled water was added to 75 g tempeh powder and the suspension was stirred 1 h at room temperature. The pH of the suspension was kept at 8.0 through the addition of NaOH 2 M for every 30 min. The suspension was centrifuged (30 min, 10000× *g*, 20 ºC) and filtered to obtain crude extract. Following lyophilization for 96 h, the extract was stored at 4 ºC before analysis.

2.4 Extraction of Bacterial DNA

Total bacterial DNAs from tempeh samples were extracted based on the method described by Seumahu et al. (2013) with modifications. Phosphate buffer saline (PBS) pH 7.4 (150 mL) was added to 50 g of diced tempeh. The mixture was homogenized and the suspension was centrifuged at 1000× g for 10 min. The collected supernatant was centrifuged again at 10000× g for 10 min. The pellets were collected for bacterial DNA extraction using ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, Orange, CA, USA) and the DNA isolate was kept at -20 ºC.

2.5 Measurement of Anti-adhesion Bioactivity against ETEC

Anti-adhesion bioactivity of tempeh extract against ETEC adhesion to eukaryotic cells was measured using yeast agglutination assay based on the method described by Mirelman et al. (1980). *S. cerevisiae* was grown in potato dextrose broth (HiMedia, India) at 37 ºC for overnight with shaking. ETEC was grown overnight without shaking in Luria Bertani broth (HiMedia) at 37 ºC. Both cell cultures were centrifuged at 3000× *g* for 5 min at 4 ºC and the collected pellets were suspended in an equal volume of PBS pH 7.4. The cells were washed twice in PBS pH 7.4 and the densities of yeast and ETEC suspensions were adjusted to OD_{600} of 1.0 and 0.5, respectively.

Tempeh extract was suspended in PBS pH 7.4 to the concentration of 2 % (w/v) and the suspension was vortexed for 30 min. The suspension was centrifuged at 10000× *g* for 10 min and the supernatant was collected. Mannose 2 % (w/v) in PBS pH 7.4 was used as a positive control. Tempeh extract was mixed with ETEC suspension with the ratio of 1:1 in a 96-wells microtiter plate. The mixture was incubated at room temperature with orbital shaking for 10 min. Afterwards, yeast suspension at the same volume was added into the mixture and followed with incubation for 30 min with orbital shaking. A mixture of an equal volume of yeast, ETEC, and PBS pH 7.4 was used as a negative control. The suspension was transferred onto concave object glass and covered with cover glass. Cell agglutinates were observed using a light microscope (Nikon Eclipse E100; Tokyo, Japan) at 100× magnification. The number of yeast agglutinates was enumerated using the program DinoCapture 2.0 (Dino-Lite, Torrance, CA, USA).

The number of agglutinates was determined as the sum of agglutinates observed from seven location points and the measurement was done in triplicates. Anti-adhesion bioactivity of tempeh extract against ETEC adhesion to yeast cells was expressed as the percent of adhesion inhibition which was calculated with the following formula (1).

$$\%\ adhesion\ inhibition = 100\% - \frac{\bar{X}N}{\bar{X}C} \times 100\% \ldots (1)$$

Whereas:
$\bar{X}N$ = average number of agglutinates in sample
$\bar{X}C$ = average number of agglutinates in negative control

2.6 Bacterial Quantification with RT-PCR

Quantification of total bacteria, Firmicutes, and γ-Proteobacteria was carried out using RT-PCR based on 16s rDNA that are specific to each targeted bacterial group. Bacterial genomes of *E. coli*, *S. aureus*, and *S. enterica* ATCC 51741 were extracted using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) to generate standard curves for total bacteria, Firmicutes, and γ-Proteobacteria, respectively. DNA isolates from pure bacterial culture were amplified based on the method described by Soka et al. (2014) and the amplified products were diluted tenfold to seven standard concentrations between 10^2 – 10^{10} DNA copy/mL. Samples and standards were each added into PCR mix comprised of 10 µL Solg™ Real-Time PCR Smart Mix (SolGent, Daejeon, South Korea), 1 µL DNA template, 1 µL of each primer (10 pmol.μL^{-1}) (Table 1) and NFW for the total volume of 20 µL. The primers were designed specifically to amplify the regions of 16s rDNA that are specific to each of the bacterial groups used in this experiment. The PCR reaction conditions were as follow: 94 ºC for 5 min; 40 cycles of 94 ºC for 20 sec, 55 – 57 ºC (Table 1) for 20 sec and 72 ºC for 50 sec; and 72 ºC for 15 sec. Each sample was amplified in triplicate. RT-PCR reading of the standards showed linearity between C_t value and log of DNA copy number ($R^2 > 0.99$). The concentration of a certain bacterial group in a sample was determined based on the C_t value and regression equation generated from the standard curve. All of the measurements were done in triplicates.

2.7 Statistical Analysis

The data were statistically analyzed using SPSS Statistics (IBM Corporation, Armonk, NY, USA). The correlation between bacterial numbers based on RT-PCR and anti-adhesion bioactivity of tempeh extract was determined by calculating the *P*-value and Pearson correlation coefficient (*R*-value) of the two variables. Correlation was considered significant at $P < 0.01$ and strong at $R > 0.70$.

Table 1. Primers for the amplification of group-specific 16s rRNA gene.

Target bacterial group	Primer	Sequence (5'-3')	Size (bp)	Annealing temperature (°C)	Citation
Universal bacteria	Eub338F	ACTCCTACGGGAGGCAGCAG	220	57	(Soka et al., 2014)
	Eub518R	ATTACCGCGGCTGCTGG			
Firmicutes	Firm934F	GGAGTATGTGGTTTAATTCGAAGCA	126	56.5	(Guo et al., 2008)
	Firm1060R	AGCTGACGACAACCATGCAC			
γ-Proteobacteria	1080γF	TCGTCAGCTCGTGTYGTGA	122	55	(Karamipour et al., 2016)
	γ1202R	CGTAAGGGCCATGATG			

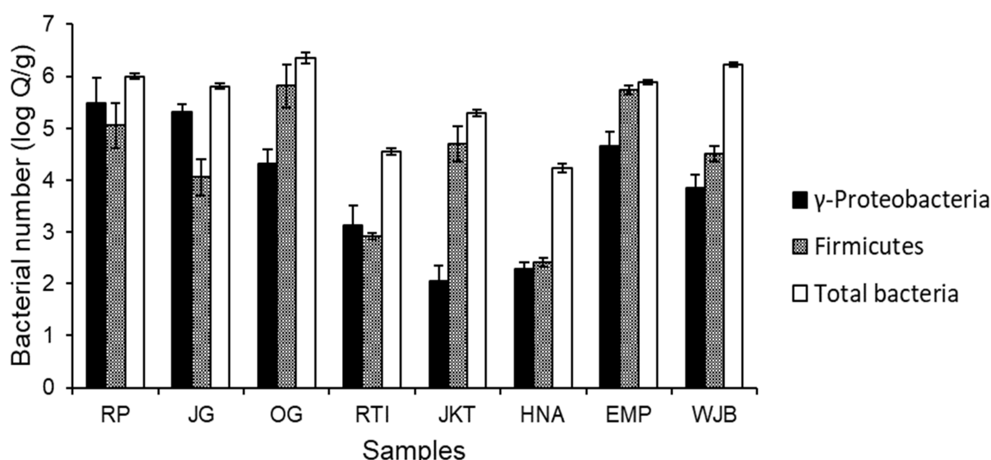

Figure 2: Anti-adhesion bioactivity of 2 % (w/v) tempeh extract against ETEC adhesion to *S. cerevisiae*. Mannose (2 % (w/v)) was used as a positive control. Bars represent the mean of % adhesion inhibition based on triplicates. Error bars represent standard errors.

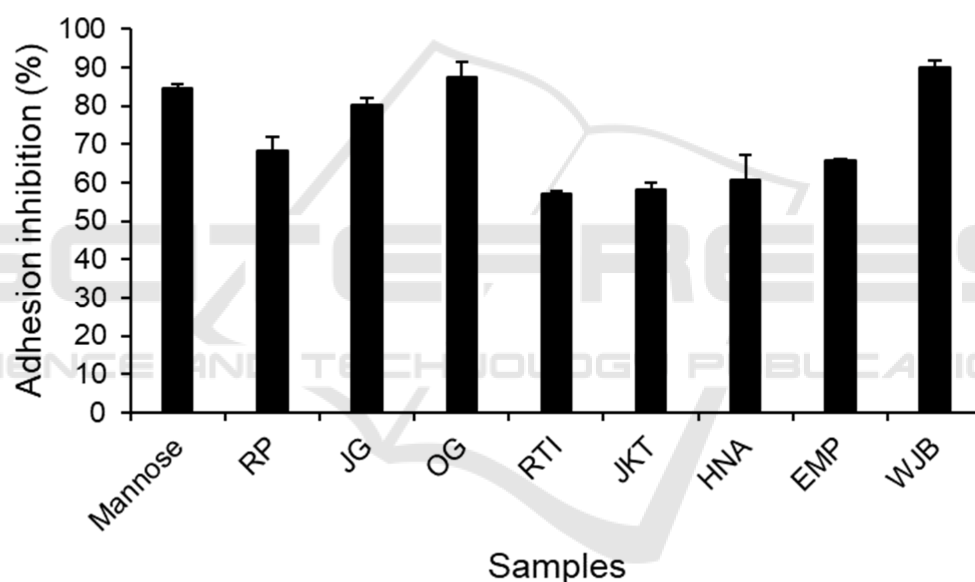

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Figure 3: Bacterial amount in tempeh samples based on RT-PCR analysis with primers that amplified regions of 16s rDNA that are specific to γ-Proteobacteria, Firmicutes and total bacteria. Bars represent mean values, expressed as log copy number per gram total weight of sample (log Q/g), from three replicates of measurement. Error bars represent the standard error of mean.

3 RESULTS AND DISCUSSION

3.1 Tempeh Samples Showed a Varying Level of Bacterial Abundance and Anti-adhesion Bioactivity

Yeast agglutination assay was used to determine the bioactivity of tempeh extract in inhibiting ETEC adhesion to eukaryotic cells. The yeast *S. cerevisiae* acted as a model organism for eukaryotic cells which will form agglutinates in the presence of ETEC. We quantified the number of agglutinates under a microscope with 100× magnification and compared the number of agglutinates between yeast and ETEC suspension with and without the addition of tempeh extract. Mannose was used as a positive control due to its capability to bind to ETEC fimbriae thus inhibiting yeast agglutination. Yeast and ETEC

suspension treated with mannose 2% (w/v) resulted in 84.66 ± 1.14 % reduction of agglutinates compared to untreated suspension. Figure 2 showed that tempeh samples resulted in varying levels of adhesion inhibition ranging from 57 to almost 90 %. Tempeh fermented in laboratory condition (RP, JG, and OG) tended to have anti-adhesion bioactivity compared to commercial tempeh (RTI, JKT, HNA, and EMP) except for WJB. Both extracts from OG and WJB showed anti-adhesion bioactivity higher than mannose control at 87.52 ± 4.04 % and 89.95 ± 1.84 % respectively. Overall, this varying level of anti-adhesion bioactivity was ideal for this experiment as it allowed us to plot the data against bacterial number from the next part of this experiment.

Bacterial number in tempeh samples was determined using RT-PCR to measure the number of total bacteria and the specific phyla of Firmicutes and γ-Proteobacteria. Figure 3 showed that the bacterial numbers in tempeh were also varied from one sample to another. There was less variation of total bacteria between tempeh made in the laboratory with different starter culture indicating that starter culture did not play a major role in affecting total bacterial number. Despite the lack of significant variation of total bacterial number in lab-made tempeh, there was a variation of bacterial profile composition with RP and JG containing more γ-Proteobacteria compared to OG. The bacterial profile in most samples was dominated by the phylum Firmicutes with the exception of RP, JG, and JKT that were dominated by γ-Proteobacteria. RTI and HNA contained the fewest number of bacteria at 4.55 ± 0.06 and 4.23 ± 0.08 log Q/g respectively. Both RTI and HNA are commercial samples that were produced using a standardized industrial method in a hygienic condition.

3.2 Tempeh Samples Showed a Varying Level of Bacterial Abundance and Anti-adhesion Bioactivity

We plotted the anti-adhesion bioactivity against ETEC measured using yeast agglutination assay with the bacterial number in tempeh determined by RT-PCR. Figure 4A showed that there was a strong correlation between anti-adhesion bioactivity with a total bacterial number in tempeh with the *R*-value of 0.733. The correlation was very significant at $P < 0.01$. This indicated that the anti-adhesion bioactivity of tempeh against ETEC tends to increase following an increase in the number of total bacteria in the product. The correlation between anti-adhesion bioactivity and the quantity of both Firmicutes and γ-Proteobacteria was also significant at $P < 0.05$. However, the correlation of both phyla with anti-adhesion bioactivity was weak with the *R*-value of 0.446 and 0.488 for Firmicutes and γ-Proteobacteria respectively (Figure 4B and 4C). Our finding indicated that the influence of major bacterial phyla in tempeh on its anti-adhesion bioactivity against ETEC was minimal compared to the influence from bacterial community as a whole.

3.3 General Discussion

The anti-adhesion bioactivity of tempeh extract against ETEC adhesion to eukaryotic cells arises from the degradation of soy matrix polysaccharide into bioactive oligosaccharides (Roubos-van den Hil et al., 2010). This bioactivity is not exclusive to soybean fermentation by fungal culture (Roubos-van den Hil et al., 2010). Bacterial fermentation of soybeans resulted in similar anti-adhesion bioactivity against ETEC. We found the indication that bacterial role in the release of bioactive oligosaccharides is also present in tempeh fermentation by fungal inoculum.

In this experiment, we focused on two specific phyla: Firmicutes and γ-Proteobacteria. Both phyla are the major bacterial groups reported in commercial tempeh that are available in Indonesia (Radita et al., 2017). We hypothesized there could be two mechanisms on how the bacterial population can contribute to the increase of anti-adhesion bioactivity in tempeh. First, bacteria could break down soy matrix polysaccharide thus making it more accessible for further breakdown by fungi or vice versa. Second, the bacteria produce bacterial exopolysaccharides (EPS) that can bind to ETEC cells.

It has been reported that most lactic acid bacteria (LAB) are capable of producing bioactive EPS (Welman & Maddox, 2003) and these LAB are also known to be present in tempeh (Radita et al., 2018). If the latter assumption was true, there should be a correlation between the Firmicutes population with anti-adhesion bioactivity. However, the absence of correlation seemed to indicate that bacterial role on anti-adhesion bioactivity was more likely due to the breakdown of soy matrix polysaccharide. We would like to mention that our finding did not negate the possibility of EPS or other bacterial secondary metabolites playing a role on anti-adhesion bioactivity in tempeh. This research was only focused on the two major phyla in tempeh and it is possible that other minor phyla could be more strongly correlated to anti-adhesion bioactivity. Extraction and quantification of EPS could also provide more definitive information on its bioactive potential.

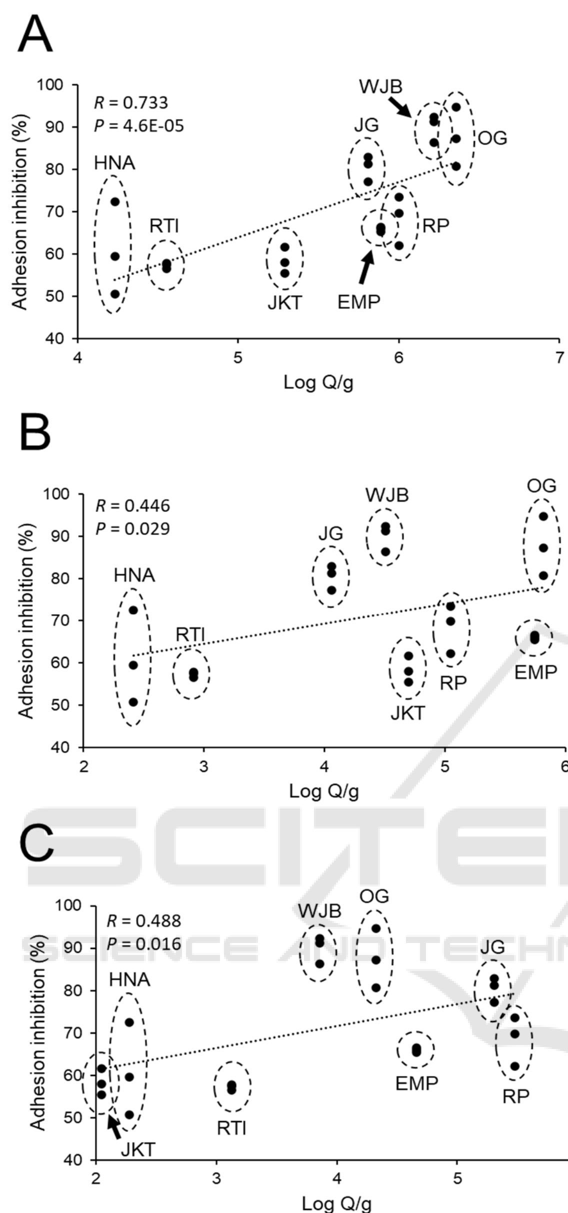

Figure 4: Correlation between anti-adhesion bioactivity of tempeh extract at 2 % (w/v) (expressed as % adhesion inhibition) and bacterial number in tempeh based RT-PCR analysis with primers that amplified regions of 16s rDNA that are specific to A) total bacteria, B) Firmicutes and C) γ-Proteobacteria. Regression line is represented by a dotted line. Correlation between anti-adhesion bioactivity and the number of each bacterial group was expressed as P-value to indicate correlation strength and R-value to indicate correlation significance.

Although we found that total bacterial number is more strongly correlated to anti-adhesion bioactivity, the role of the bacterial profile could still not be crossed out. Figure 2 showed that there was a stark difference in the anti-adhesion bioactivity of tempeh extract from three lab-made tempeh against ETEC. The three samples were produced from the same batch of soybeans and through the same line of preparation with the only difference was after they were inoculated with different starter and incubated separately at the same temperature. It has been reported that variation in starter culture does not affect the total bacterial number in tempeh (Pramudito et al., 2021; Radita et al., 2017). Figure 3 showed that the number of total bacteria based on RT-PCR was not too varied especially in the case of RP and JG.

The contrast between the variation of anti-adhesion bioactivity and total bacterial number among the three lab-made samples could imply that bacterial composition might still play a role in anti-adhesion bioactivity. A previous report mentioned that although variation in tempeh starter did not affect the total bacterial population, it could still influence bacterial composition in the final product (Pramudito et al., 2021). JG was made with the commercial starter ‘Cap Jago’ that has been reported to result in a lower rate of fungal mycelium growth thus allowing spoilage bacteria, mainly from the phylum γ-Proteobacteria, to grow uninhibited in the early stage of fermentation (Pramudito et al., 2021). The rapid growth of γ-Proteobacteria during the fermentation process could lead to more degradation of soy matrix polysaccharides. Bacteria from the phylum γ-Proteobacteria such as the genus *Pseudomonas* is known to be capable of producing polysaccharide-degrading enzymes such as glycoside hydrolase (Edwards et al., 2010; Kurakata et al., 2008). More research is needed to see the role of specific bacterial growth dynamic on the formation of bioactive oligosaccharides in tempeh.

4 CONCLUSIONS

The amount of total bacteria in tempeh is strongly correlated to the anti-adhesion bioactivity of tempeh extract against ETEC adhesion to eukaryotic cells. However, there was only a weak correlation between anti-adhesion bioactivity with the amount of two major bacterial phyla in tempeh, Firmicutes, and γ-Proteobacteria. Our finding did not rule out the possibility that a specific bacterial phylum could still influence anti-adhesion bioactive in tempeh through bacterial growth dynamic during the fermentation process. Results from this experiment could provide new insight on the development of tempeh into a functional food product for diarrhea prevention. The

bacterial amount and profile present during tempeh fermentation process need to be considered to produce tempeh with optimum bioactive potential against ETEC adhesion.

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