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FULL PAPER

Positive correlation between the number of bacteria in soybean tempeh with the bioactivity of its extract against enterotoxigenic *Escherichia coli* (ETEC) adhesion to eukaryotic cells

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Abstract

Bioactive oligosaccharides from soybean tempeh can inhibit the adhesion of enterotoxigenic *Escherichia coli* (ETEC) to intestinal cells, thus reducing the severity of ETEC-mediated diarrhea. Bacteria are also present in tempeh but there has yet been any report regarding their effect on the anti-adhesion bioactivity of tempeh. In this research, the bacterial number in tempeh was quantified and the anti-adhesion bioactivity of tempeh extract was determined using yeast agglutination assay. Statistical analysis showed a moderately (R = 0.69) significant positive correlation (P < 0.01) between the number of bacteria in tempeh and the anti-adhesion bioactivity of its extract. In conclusion, tempeh that contains more bacteria is more effective in inhibiting ETEC adhesion to eukaryotic cells. This could be due to a symbiosis between *Rhizopus* and bacteria in breaking down soy matrix polysaccharides to release bioactive oligosaccharides.

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is associated with two types of diarrhoea: weanling diarrhoea among children and traveller's diarrhoea (Qadri *et al.*, 2005). The former is considered a major problem in developing countries such as Indonesia where food hygiene is lacking. The disease is triggered by the adhesion of ETEC to the intestinal epithelium, which leads to the colonization and infection of the intestinal tract. ETEC adhesion is also required for enterotoxins production that leads to an increase in water secretion and a decrease in nutrient absorption by enterocytes (Nagy and Fekete, 2005).

Tempeh is a traditional Javanese fermented food made from the fermentation of soybean by moulds such as *Rhizopus oligosporus*. The major steps of tempeh production include soybean boiling, soaking, dehulling, inoculation with starter and incubation to allow for mould growth and fermentation. This fermentation process contributes to texture and flavour development and inactivates antinutritional components in soybeans (Nout and Kiers, 2005). Moreover, it has been reported that tempeh could decrease diarrheal severity compared to unfermented toasted soybeans (Kiers *et al.*, 2007). This bioactivity arises from oligosaccharide molecules >30 kDa in molecular mass that might be released from the degradation of soy matrix polysaccharides by microorganisms. These bioactive oligosaccharides are known to contain arabinose residue and are stable in high temperatures (Roubos-van den Hil, Schols, Nout *et al.*, 2010).

Bacteria are also present in tempeh and can determine some of the product's characteristics. The bacterial population in tempeh is dominated by Firmicutes, especially lactic acid bacteria (LAB), with Proteobacteria as the sub-dominant phylum (Radita *et al.*, 2018). Radita *et al.* (2017) reported that the bacterial population in tempeh, in particular LAB, increased significantly over the course of soybean soaking. During soaking, the pH of soybeans and their soaking water decrease due to the production of organic acids by LAB (Nout *et al.*, 1987). Such low pH helps to inhibit the growth of spoilage microorganisms. Bacteria also influence the nutritional and sensory properties of tempeh such as through the production of vitamin B12 by *Klebsiella pneumoniae* and the formation of bitter flavour by *Escherichia coli* (Keuth and Bisping, 1994; Barus *et al.*, 2008; Ayu *et al.*, 2014; A'yun *et al.*, 2015). The bacterial population in tempeh could also act as an immunostimulant which can help to protect against

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pathogen infection (Soka *et al.*, 2014).

Despite the potential of tempeh as a functional food with anti-diarrheal activity, to date, there are no reports regarding the effect of bacterial number in tempeh on its bioactivity. Most findings were based on tempeh fermented in laboratory conditions, which did not consider the presence of these bacterial populations. Considering that the bioactive compound content in tempeh might arise from the degradation of soy polysaccharides, it is possible that these bacteria might play an important role as well. Therefore, this research is aimed to study the correlation between the bacterial number and anti-adhesion bioactivity in tempeh using the yeast agglutination assay, in which the yeast *Saccharomyces cerevisiae* acted as a substitute to epithelial cells to model ETEC adhesion to eukaryotic cells. Findings in this research will shed light on the ideal tempeh microbial profile that can inhibit ETEC infection, in search of an accessible prebiotic product against ETEC-induced diarrhoea.

2. Materials and methods

2.1 Materials

Commercial tempeh samples were from five different producers in Bogor (EMP and RTI), Jakarta (JKT) and Surabaya (HNA). All samples were stored and transported in an icebox prior to analysis. In the case of HNA, tempeh was kept at room temperature for 48 – 72 hrs until fungal mycelium was fully formed before it was stored in an icebox. Table 1 shows a summary of various production methods involved for each commercial tempeh. All tempeh samples were made with the same type of yellow-seeded elongated soybean. Starters used for lab-made tempeh were the commercial starters Raprima (RP; PT. Aneka Fermentasi Industri, Bandung, Indonesia), Cap Jago (JG; UD. Jaya Mulya, Kediri, Indonesia) and cassava-based *onggok* (by-product of tapioca production) starter (OG; acquired from a traditional producer in Cisauk, Banten, Indonesia). *Saccharomyces cerevisiae* and ETEC cultures were obtained from the Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia (Cisauk, Indonesia). D-(+)-mannose was purchased from Sigma Aldrich (Darmstadt, Germany).

2.2 Tempeh preparation

Lab-made tempeh was prepared based on a method described by Kustyawati (2009) with modifications. Full -fat yellow-seeded elongated soybeans were used for tempeh fermentation and purchased from Pasar Modern Intermoda BSD (Tangerang, West Java). The soybeans were soaked overnight in distilled water at room temperature and boiled and dehulled. After the soybeans were cooled and dried, they were mixed thoroughly with a tempeh starter (0.2% (w/w) of soybeans). The mixture was packed in a perforated plastic bag and incubated at 30^{o}C for 48 hrs. The resulting tempeh was immediately used for analysis.

2.3 Bacterial enumeration

Bacteria were isolated from the inner part of tempeh, in which the outer surface of tempeh samples was sliced off, and the remaining samples were diced. Diced tempeh (10 g) was transferred into a plastic bag and 90 mL phosphate-buffered saline (PBS) pH 7.4 (Ambion, USA) was added to the sample. The mixture was homogenized using a stomacher for 1 min. The bacterial suspension was collected in a centrifuge tube to be immediately used for bacterial enumeration. Serial dilutions of bacterial suspensions up to 10^{-8} followed by plate count agar (Oxoid, USA) were poured into a petri dish and incubated at 30^{o}C for 48 hrs. The measurement was replicated four times for each dilution factor and the bacterial number was expressed as log CFU/g tempeh.

2.4 Extraction of bioactive oligosaccharides

Bioactive oligosaccharides were extracted from tempeh using a method as described by Roubos-van den Hil, Schols, Nout *et al*. (2010) with modifications. Commercial and lab-made tempeh were lyophilized for 96 hrs and ground into a fine powder. The powder (75 g) was stirred into 1 L distilled water for 1 hr at room temperature. The pH of the suspension was adjusted to 8.0 with NaOH 2 M every 30 mins. The suspension was then centrifuged (30 mins, 10000×*g*, 20^{o}C) and filtered to obtain soluble tempeh crude extract, which was

Table 1. Different production methods for commercial and lab-made tempeh samples

Sample	Source	Cooking	Dehulling	Starter mixing	Packaging	Environmental hygiene	Water source
EMP	Bogor	One stage	Mechanic	Manual	Banana leaf	Non-aseptic	Groundwater
RTI	Bogor	Two stages	Mechanic	Mechanic	Plastic	Aseptic	PAM[1]
JKT	Jakarta	Two stages	Mechanic	Manual	Plastic	Semi-aseptic	PAM
HNA[2]	Surabaya	Two stages	Mechanic	Mechanic	Plastic	Aseptic	PAM
Lab-made[3]	Cisauk	One stage	Manual	Manual	Plastic	Aseptic	PAM

[1]Water provided by the national water utility service.
[2]HNA was distributed to the consumers in the state of mid-fermentation to ensure tempeh freshness.
[3]Made with three different commercial starters.

further lyophilized for 96 hrs and stored at 4°C before analysis.

2.5 Determining the anti-adhesion bioactivity of samples

The capability of tempeh extract to inhibit ETEC adhesion to eukaryotic cells was analysed using yeast agglutination assay as described by Mirelman *et al.* (1980) with modifications. Tempeh extract (2% (w/v)) was suspended in PBS pH 7.4 and vortexed for 30 mins to ensure optimum homogenization. The suspension was centrifuged at 10000×*g* for 10 mins and the supernatant was collected. Mannose 2% (w/v) was used as positive control.

Saccharomyces cerevisiae cells were grown on potato dextrose broth (HiMedia, India) with shaking overnight at 30°C. ETEC was grown on Luria Bertani broth (HiMedia) without shaking at 37°C for 20 hrs. Both yeast and ETEC cells were pelleted by centrifugation at 3000×*g* for 5 mins at 4°C and suspended in an equal volume of PBS pH 7.4. The cells were washed twice in PBS pH 7.4 and cell densities of yeast and ETEC suspensions were adjusted to OD_{600} of 1.0 and 0.5, respectively. Aliquots of the tempeh extract (10 µL) were mixed with ETEC suspension (10 µL) in a 96-wells microtiter plate and incubated at room temperature with orbital shaking at 300 rpm for 10 mins. Yeast suspension (10 µL) was later added into the mixture and incubated for 30 mins at 300 rpm. A mixture of an equal volume of yeast and ETEC suspension in PBS pH 7.4 was used as a negative control. The suspension was then transferred onto concave object glass and carefully covered with cover glass. Cell agglutinates were observed using a light microscope (Nikon Eclipse E100; Tokyo, Japan) at 100× magnification. The program DinoCapture 2.0 (Dino-Lite, Torrance, CA, USA) was used for the enumeration of yeast agglutinates. The number of agglutinates was determined as the sum of agglutinates observed from seven location points in the object-glass and the measurement was repeated six times. Anti-adhesion bioactivity of samples against ETEC adhesion to yeast cells was calculated with the following formula:

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

Where $\bar{X}N$ is the average number of agglutinates in sample and $\bar{X}C$ is the average number of agglutinates in negative control.

2.6 Statistical analysis

SPSS Statistics (IBM Corporation, Armonk, NY, USA) was used for statistical analysis of all data acquired from this experiment. The anti-adhesion bioactivity of tempeh extract was compared to the positive control (mannose 2% (w/v)) using one-way ANOVA and two groups were considered statistically different at $P < 0.05$. The correlation between bacterial number in tempeh based on TPC and anti-adhesion bioactivity of tempeh extract was determined by calculating *P*-value and Pearson correlation coefficient (*R*-value) of the two variables. Correlation was considered significant at $P < 0.01$.

3. Results

3.1 Bacterial number in tempeh samples

TPC showed that the bacterial number in tempeh samples among commercial samples was more varied compared to that among lab-made tempeh (Figure 1). EMP contained the highest bacterial number among commercial samples at 9.24±0.004 Log CFU/g, while RTI contained the least number of bacteria compared to the other samples at 6.42±0.029 Log CFU/g. The bacterial number in tempeh made using a two-stage cooking process (RTI, JKT and HNA) was lower than in tempeh made with a one-stage cooking process (EMP and lab-made samples). The number of bacteria in lab-made tempeh (OG, RP and JG) was similar to that in EMP and higher compared to other commercial tempeh. The abundance of bacteria in lab-made tempeh could be due to the production method, in which one-stage cooking was employed and the soybeans were dehulled manually. Lab-made tempeh with the highest and lowest bacterial number was JG (9.61±0.006 log CFU/g) and OG (9.23±0.027 log CFU/g), respectively. All lab-made tempeh was prepared using the identical method, except for the type of starter culture used, thus resulting in samples with a similar level of bacteria. In general, the

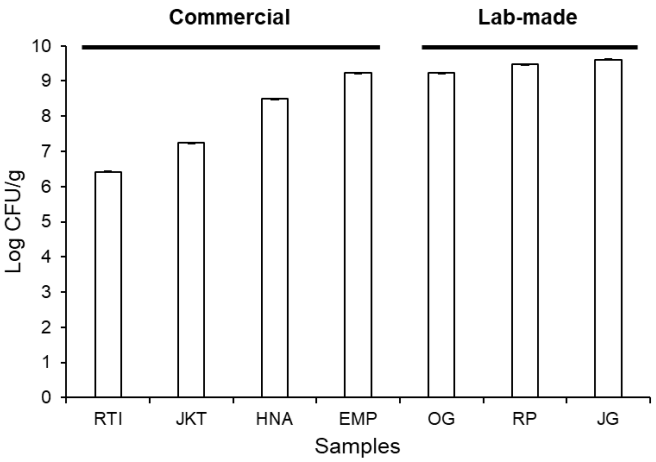

Figure 1. The bacterial enumeration in commercial (RTI, JKT, HNA and EMP) and lab-made (OG, RP and JG) tempeh samples based on total plate count. Bacterial colonies were grown on plate count agar (Oxoid) and counted after 48 hrs incubation at 30oC. Bacterial count is expressed at log CFU/g total weight of sample based on four replicates. Error bars represent standard errors.

samples were found to be suitable for correlation study as the set contained samples with both varying and similar degrees of bacterial number.

3.2 Anti-adhesion bioactivity of tempeh extract

The anti-adhesion bioactivity of tempeh extract against ETEC was measured using yeast agglutination assay. In the presence of ETEC, yeast cells formed agglutinates due to the adhesion of ETEC cells to the surface of the yeast cell wall. Carbohydrates such as mannose bind to ETEC fimbriae thus inhibiting the formation of cell agglutinates (Figure 2A). In this research, the number of cells agglutinates quantified were observed at 100× magnification to determine the anti-adhesion bioactivity of the sample. The anti-adhesion bioactivity of tempeh extract at 2% (w/v) was expressed as percent adhesion inhibition by comparing the number of agglutinates in yeast and ETEC suspension added with tempeh extract, to suspension without tempeh extract addition. Mannose 2% (w/v) was used as positive control and showed 81.73±2.57% adhesion inhibition. In general, extracts from lab-made tempeh showed higher adhesion inhibition against ETEC than commercial samples (Figure 2B). All samples showed significantly lower bioactivity (P < 0.05) than mannose at the same concentration, except for OG and JG. Among commercial tempeh, EMP extract had the highest bioactivity at 64.53±0.67% inhibition. RTI extract showed the lowest inhibition against ETEC adhesion at 51.37±2.82%. The adhesion inhibition bioactivities of lab-made JG and OG were not significantly different compared to mannose at 85.36±2.37 and 88.41±1.88%, respectively. RP was the only lab-made tempeh that showed a significantly lower adhesion inhibition level compared to mannose at 65.59±1.99%.

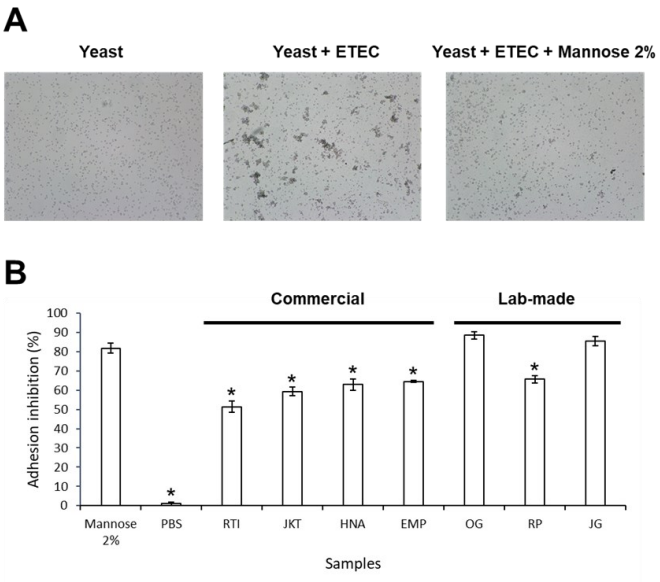

Figure 2. Yeast agglutination assay for the determination of anti-adhesion bioactivity against ETEC. A) Microscopic observation of *S. cerevisiae* cells at 100× magnification. From left to right: *S. cerevisiae* cells with no treatment, in the presence of ETEC and the presence of ETEC and 2% (w/v) mannose. B) 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 and phosphate buffer saline (PBS) pH 7.4 was used as a negative control. Bars represent the mean of percentage adhesion inhibition based on six replicates. Error bars represent standard errors. Bars with asterisks notation indicate significant difference (P < 0.05) between tempeh treatment and the mannose control.

3.3 Bacterial number in tempeh positively correlated with anti-adhesion bioactivity of its extract

Statistical analysis showed a significant correlation between the bacterial number in tempeh and its adhesion inhibition bioactivity (P<0.01). As the number of bacteria in tempeh increased, the anti-adhesion effect was also higher (Figure 3). Furthermore, both variables had a Pearson correlation coefficient (R-value) of 0.69, indicating a strong positive relationship between them.

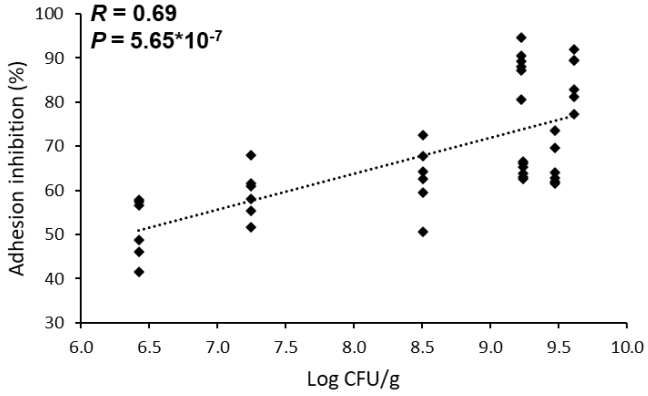

Figure 3. Correlation between anti-adhesion bioactivity of tempeh extract at 2% (w/v) (expressed as% adhesion inhibition) and bacterial number in tempeh based on total plate count (expressed as Log CFU/g tempeh). Regression line is represented by a dotted line. Statistical analysis with SPSS Statistics (IBM Corporation) gave the P-value of 5.65×10^{-7} and R-value of 0.69, indicating a significant positive moderate correlation (P < 0.01).

4. Discussion

In this research, seven tempeh samples that consisted of four commercial and three lab-made tempeh were analysed. The commercial tempeh was obtained from four different producers employing various production methods (Table 1). This resulted in a highly variable bacterial number among commercial samples (Figure 1). Samples that were produced with the two-stage cooking process (RTI, JKT and HNA), contained a lower number of bacteria compared to EMP. The two-stage cooking process is known to decrease the bacterial number in the product compared to one-stage cooking (Mulyowidarso *et al.*, 1989). RTI was also produced with a shorter

soaking time compared to other commercial tempeh (Muzdalifah *et al.*, 2017), which might also contribute to the low bacterial number in the product. Mulyowidarso *et al*. (1989) reported that the number of bacteria in soybean peaked after 24 hrs of soaking thus shorter soaking time could significantly impact the number of bacteria in RTI. In the two-stage cooking process, soybeans were cooked before and after the soaking step (Barus *et al.*, 2008; Efriwati *et al.*, 2013). EMP and lab-made tempeh, on the other hand, were prepared using a one-stage cooking process. It should be noted that the two-stage cooking process is still an uncommon practice in Indonesia with only one in five producers practising the method (Barus *et al.*, 2008).

Similar to RTI, HNA was produced in a hygienic indoor environment, yet the number of bacteria in HNA was the second-highest among commercial samples. This could be related to how this tempeh was distributed, in which it was sold to consumers even before the fermentation process has been completed to ensure product freshness. Such an approach might favour bacterial growth as *Rhizopus* might grow slower due to less ideal fermentation conditions during distribution. It has previously been reported that slower fungal growth during tempeh fermentation led to an increase in bacterial count (Mulyowidarso *et al.*, 1990). It should also be noted that there were contradictory findings regarding the effect of the cooking method on specific bacterial populations, i.e. LAB. Efriwati *et al*. (2013) reported a higher LAB number in tempeh made with the one-stage cooking process, while in contrast, Nurdini *et al*. (2015) reported that the LAB population was higher in tempeh made with two-stage cooking. This indicated that the bacterial population in tempeh could be very dynamic and incrementally influenced by various small parameters (Radita *et al.*, 2018). Many conditions tend to be difficult to control, resulting in a product with a different bacterial profile from time to time.

In the case of EMP, soybeans were cooked only once before soaking. The high bacterial number in EMP has previously been reported and could be due to bacterial contamination after cooking and before the addition of tempeh starter (Barus *et al.*, 2008; Seumahu *et al.*, 2013; Radita *et al.*, 2017; Radita *et al.*, 2018). EMP was produced traditionally in an open-aired environment and the soybean soaking process was done using groundwater (Seumahu *et al.*, 2013). Other samples in this experiment were made using soybeans soaked in fresh water provided by the Indonesian water utility service.

The high bacterial number in lab-made tempeh could be due to the growth of endogenous bacteria in soybeans. For lab-made tempeh, soybeans were cooked once after the soaking process, a step that was reported to decrease only an insignificant number from the plethora of bacteria that grew during the soaking process (Mulyowidarso *et al.*, 1989). Compared to commercial samples, the bacterial number in lab-made samples was relatively similar to one another. All three lab-made tempeh were produced with identical methods, with the type of starter used being the only variable factor. The lack of variation in bacterial number was in accordance with a previous report that showed that starter type did not significantly influence the bacterial profile in tempeh, as opposed to other parameters such as the soaking process (Radita *et al.*, 2017).

The anti-adhesion bioactivity of tempeh extracts tended to be lower than that of mannose at the same concentration, except for OG and JG (Figure 2B). Both OG and JG showed anti-adhesion bioactivity that was not significantly different from mannose. The bioactivity of OG and JG was also 20% higher compared to RP, another lab-made sample. RP and JG were made using commercial starters, namely Raprima and Cap Jago, respectively. In Indonesia, Raprima is a commercial tempeh starter that is commonly used for tempeh production in West and Central Java (Radita *et al.*, 2017). Cap Jago is another commercial starter which uses is limited to East Java. The slow rate of mycelium formation has been reported in tempeh produced with Cap Jago starter, resulting in tempeh with a less cohesive structure (Amaliyah *et al.*, 2018). The slow rate of fungal growth might also allow for higher bacterial activity, especially during the early stage of the fermentation, as bacteria would have more time to utilize the nutrition in soybeans before having to compete with fully grown mould. OG was made using cassava-based *onggok* starter. *Onggok* refers to a cassava by-product from tapioca production (Radita *et al.*, 2017). To make *onggok* starter, cassava is pressed into a cake, warmed up and used as a growth medium for *Rhizopus* before it is dried and ground into powder (Owens, 2014; Radita *et al.*, 2017). The use of *onggok*--based starters is often limited to specific producers only, and each producer may have different production methods. In addition, the microbial profile in *onggok* is richer and more diverse as the starter is often produced using the uncontrolled traditional method (Radita *et al.*, 2017). During *onggok* production, the cassava cake was not cooked and it was treated with warm water, which would an ideal environment for bacterial growth and metabolism (Owens, 2014; Tamam *et al.*, 2019). This might lead to the presence of more active bacteria in the starter, which might account for higher bioactive oligosaccharide content in tempeh.

This research showed a significant positive

correlation between the number of bacteria in tempeh and the anti-adhesion bioactivity of tempeh extract against ETEC. This finding could provide a new paradigm for the development of tempeh as a functional product. Roubos-van den Hil, Schols, Nout *et al*. (2010) reported that the anti-adhesion bioactivity in tempeh was due to the presence of oligosaccharides derived from the breakdown of soy matrix polysaccharides by *Rhizopus*. The presence of arabinose residues might play an important role in determining the anti-adhesion bioactivity of the product. Thus far, there has yet been any report that considered the effect of bacterial number in tempeh on its anti-adhesion bioactivity. Roubos-van den Hil, Nout, van der Meulen *et al*. (2010) showed that the bacterial fermentation of soybean, such as by *Bacillus* spp. increased its bioactivity. However, the report did not explore the possibility of bacterial role on bioactivity in real conditions for tempeh fermentation because the samples were made in a controlled laboratory environment. Our study is the first to show a tendency of anti-adhesion bioactivity increase following the increase of bacterial count in tempeh.

Some bacteria can produce polysaccharide-degrading enzymes such as exo-glucanase and β-glucosidase (Koeck *et al.*, 2014; Asha *et al.*, 2015). A higher bacterial count might allow for higher production of enzymes that are required to break down complex polysaccharides in tempeh into bioactive oligosaccharides. This increase could be due to mould-bacteria symbiosis during fermentation. It is possible that bacterial polysaccharide-degrading enzymes helped the initial breakdown of complex polysaccharides in the soybean matrix, therefore increasing their accessibility for the mould. The opposite might also take place where bacteria provide an extra step in the degradation of oligosaccharides released by moulds, thus increasing their bioactivity. *Bacillus subtilis* found in tempeh was reported to release arabinan-degrading enzymes (Kaji and Saheki, 1975; Raposo *et al.*, 2004), and this might lead to an increase in arabinose-rich oligosaccharides. Kiers *et al*. (2002) reported that the growth of *B. subtilis* was not hindered by *Rhizopus* indicating that the modulation of *B. subtilis* might be a promising strategy to develop tempeh with higher anti-adhesion bioactivity.

Bacterial exo-polysaccharide (EPS) might also contribute to the increase of anti-adhesion bioactivity in samples with a high bacterial number. Bacterial EPS is accumulated by a wide range of bacterial groups, including LAB. EPS produced by *Lactobacillus reuteri*, for example, inhibited the agglutination of erythrocytes by ETEC (Wang *et al*., 2010). *Lactobacillus reuteri* had also been reported to be present in tempeh (Feng *et al.*, 2005), although the EPS-producing capability of the specific strain found in tempeh has not been investigated. González-Ortiz *et al*. (2014) have reported the inhibition of ETEC adhesion onto porcine intestinal cells by EPS. Interestingly, said EPS consisted of glucose homo-polysaccharides, indicating that a simple carbohydrate structure could also pose a bioactivity effect against ETEC adhesion. However, it has been demonstrated that tempeh fermented with *Lactobacillus plantarum* did not show anti-adhesion bioactivity against ETEC (Roubos-van den Hil, Nout, van der Meulen *et al*., 2010). *Lactobacillus plantarum* was shown to produce bioactive bacterial EPS (Dilna *et al*., 2015; Wang *et al*., 2018), yet there has been no report regarding its anti-adhesion bioactivity against ETEC. Further research needs to be done regarding the possible role of LAB in the bioactive property of tempeh.

4. Conclusion

Tempeh that contains more bacteria might be more effective in inhibiting ETEC adhesion to eukaryotic cells than tempeh that contains less bacteria. This correlation could be due to a symbiosis between *Rhizopus* and bacteria, in which one party might increase the accessibility of polysaccharides for the other, thus facilitating the production of bioactive oligosaccharides. Results from this research provide a new possibility for the exploration of the bacterial role in the bioactivity of tempeh. The next stage of this investigation should focus on the characterization of specific bacterial groups that play an important role in this correlation. Not only such development will lead to more information on the anti-adhesion bioactivity of tempeh against ETEC, but it could also lead to the development of tempeh-based functional food products.

Conflict of interest

The authors declare no conflict of interest.

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