Bio-modified soybean meal as a new protein source for food

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Abstract. Soybean meal has long been considered as a plant protein source particularly for animal feedstuff. Nowadays, there is a promising prospect of this material for food processing. However, its protein utilization has drawbacks, such as poor protein solubility and anti-nutritional factors called phytic acid. The phytic acid could be reduced by the addition of phytase, whereas nutrient content enhanced by microbial fermentation. In this study, we analyzed the bio-modified of soybean meal (SBM) through fermentation by phytase producing Lactobacillus plantarum A1-E to evaluate its effect on nutritional quality and anti-nutrient factor called phytic acid and aflatoxin. The study was designated into two treatments consisted of unfermented soybean meal (USBM) and fermented soybean meal (FSBM) by L. plantarum A1-E. Parameters observed were microbial population, phytic acid content, aflatoxin content, and nutrient composition (moisture, ash, crude protein, crude fiber, and lipid). Data were analyzed using analysis of variance (ANOVA) and a T-test to compare treatment means. FSBM by L. plantarum A1-E increased population (6.8x10⁷ CFU/ml) during 72 h incubation (P<0.05) than 0 h (2.1x10⁶ CFU/ml). FSBM reduced phytic acid content (34.13%) and aflatoxin (6.12%) compared to control (USBM). Fermentation during 72 h had on average more crude protein (1.33-fold), crude fiber (2.09-fold), ash (1.13-fold), and less crude lipid (0.71-fold) than SBM. Thus, it can be concluded that FSBM by phytase producing L. plantarum A1-E could be applied for degradation phytic acid during food processing and represents an improvement in nutritional quality thereby becomes potential raw material as a new protein source for food.

1. Introduction
Soybean meal (SBM) provide useful sources of nutrients such as complex carbohydrates, proteins (44.40%), and fiber sources (6.75%) [1]. Furthermore, SBM is known for its high protein content, balanced amino acid composition, and high level of lysine in comparison with other plant protein sources thereby it is considered as a protein source for food and animal feedstuff. However, its protein utilization has several limitations, such as undesirable anti-nutritional components caused poor protein solubility and low digestibility [2]. Among all the anti-nutritional components, phytic acid is of prime concern for human nutrition. Phytic acid is the primary storage form of both phosphate and inositol in plant seeds and presence in the form of complexes with metal cations viz. iron, zinc, and calcium cause a mineral-related deficiency in humans and also negatively impact protein and lipid utilization [3,4]. By binding plant protein, phytic acid decreased protein solubility and digestibility and the formation of phytate-carbohydrate complexes making carbohydrate less degradable thereby reduced the nutritional value of feed. Moreover, the human small intestine is a lack of a phytate-degrading enzyme in the upper part of
of the digestive tract. Therefore, the phytate-mineral complex remains partially hydrolyzed in the human gut [3]. Another anti-nutrient component in SBM was aflatoxin [5-7]. Consumption of aflatoxin-contaminated food can cause a severe health risk to humans (carcinogenic) [8].

In order to decrease its anti-nutrient factor and increase nutritional content, several approaches including physical treatment (over-heating), chemical treatment, and alcohol extraction were used. However, these methods were not fully eliminating them. For this purpose, the addition of exogenous phytase is needed. So far, no phytase product for human food applications has found its way to the market. Several researchers have reported a convincing improvement of food products by adding microbial-based phytase during food processing [9] or certain microbes for food fermentation [10,11]. Microbial-producing phytase is becoming promising approaches as inoculum for the fermentation process of SBM. *Lactobacillus plantarum* A1-E has been reported to produce extracellular phytase (41.9 U/mL) *in vitro* [12].

Solid-state fermentation (SSF) is a process in which a solid substrate is deposited in a fermenter, cultivated with single or multiple strains, and incubated in a bioreactor for a few days [13]. As a processing technology, SSF of SBM improves several physiological functions and the nutritional value, particularly protein. Fermented soybean meal (FSBM) by *Rhizopus oligosporus* removed ANF (trypsin inhibitor, phytic acid, and tannins) [14]. FSBM by microorganisms enhanced the bioavailability of nutritious components [10,11,15].

However, the application of phytase producing microbial in soybean fermentation and its effect on phytic acid content has not been reported yet. Therefore, in this study, we investigated the effect of solid-state fermentation by phytase producing *L. plantarum* A1-E on soybean meal (SBM) chemical composition and anti-nutrient factor with emphasis on phytic acid.

2. Materials and methods

2.1. Materials

The lactic acid bacteria (LAB) strain used was *L. plantarum* A1-E [12]. SBM sample was acquired from the commercial feed mill in Yogyakarta. Reagents used for microbial growth were purchased from Oxoid and chemicals for analysis were obtained from Merck.

2.2. Microbial strain and growth condition

*Lactobacillus plantarum* A1-E was grown on *de Man Rogosa* (MRS) agar media and maintained by periodic transfer. *Lactobacillus plantarum* was propagated in MRS broth media at 37 °C for 24 hours and stored at 4 °C.

2.3. Soybean meal processing

The sample of USBM was prepared by a scoop in plastic bag then grinded and homogenized to obtain particle size 1.0 mm. The sample of this SBM was subjected to fermentation process (FSBM) performed in triplicate according to Mandviwala and Khire [16] with slight modifications. Erlenmeyer flasks (250 ml) containing 10 g of SBM moistened with 10 ml distilled water were sterilized in an autoclave at 121°C for 40 min, cooled, inoculated with 5% of *L. plantarum* A1-E suspension containing 10^6 CFU/ml from a 1-day-old culture grown on MRS broth at 37 °C. The contents of each flask were mixed thoroughly with a sterile inoculating needle for uniform distribution then incubated at 37 °C for 72 h. The flask was harvested then the sample was collected for further analysis.

2.4. Microbial population and growth curve

The colony population of *L. plantarum* A1-E during fermentation was counted every 24 h using the Total Plate Count (TPC) method according to Seeley et al. [17]. One gram of FSBM was put into the falcon tube filled with 9 ml of 0.85% NaCl and homogenized then serial dilution (up to 10^5) was carried out. A total of 100 µl culture in media was taken using a micropipette and put into 900 µl of 0.85% NaCl in microtube (10^-1 dilution). Dilution then performed again to obtain 10^-2 dilution and so on. A
total of 100 µL suspension from two of the last dilution was plated into a petri dish containing MRS agar media using the spread plate method then incubated at 37 °C for 48 h. After 48 h incubation, the colony numbers that grow counted by colony counter. The colony population was calculated by the Standard Plate Count (SPC) and performed in log \(_{10}\) CFU/ml, then plotted in the growth curve.

2.5. Phytic acid determination
Phytic acid from sample was measured by the K-PHYT assay kit developed by Megazyme. The extraction process of phytic acid was carried out by the addition of one gram of sample (USBM and FSBM) in a glass beaker with 20 ml of HCl solution (0.66 M) then covered with foil and stirred vigorously overnight at room temperature. One milliliter of the extract was transferred to a microfuge tube at 13,000 rpm for 10 min. After that, immediately 0.5 ml of the resulting extract supernatant to a fresh microfuge tube and neutralized by the addition of 0.5 ml of NaOH solution (0.75 M). This neutral sample extract was used for the next step.

Enzymatic dephosphorylation reaction was subjected to two steps: free phosphorus reaction and total phosphorus reaction. Free phosphorus reaction was performed with reaction mixture consist of 0.62 ml of distilled water, 0.2 ml of solution I (buffer), 0.05 ml of sample extract) then mixed by vortex and incubated in water bath set at 40 °C for 10 min. After 10 min, the next reaction by addition of 0.02 ml of distilled water and 0.2 ml of solution 3 (ALP) then mixed by vortex and incubated in water bath set at 40 °C for 15 min. After 15 min, the reaction was stopped by addition of 0.3 ml of trichloroacetic acid (50% w/v) then centrifuged the terminated reaction at 13,000 rpm for 10 min. The supernatant was carefully transferred into fresh microfuge tube for colorimetric determination of phosphorus.

The calibration curve was prepared by making the various standard concentration of phosphorus solutions (0, 0.5, 2.5, 5, and 7.5 µg) by the addition of distilled water. One milliliter of the standard solution was added with 0.5 ml color reagent as described before then mixed by vortex and incubated in a water bath set at 40 °C for 1 h. After 1 h, mixed by vortex and transferred 1 ml to a semi-micro cuvette and read the absorbance at 655 nm.

2.6. Aflatoxin determination
Aflatoxin content was measured by the AgraQuant Total Aflatoxin Assay kit (Romer Lab) using Enzyme-linked Immunosorbent Assay (ELISA) technique. Initially, aflatoxin from USBM and FSBM samples were extracted by ground each sample so that 75% passed through a 20-mesh screen, then thoroughly mixed the subsample portion. A total of 20 g sample was added with 100 ml of 70/30 (v/v) methanol/water extraction solution then shake or blend for 3 min. The sample was kept to settle, then filtered the top layer of extract through a Whatman #1 filter, and the filtrate was collected.

Aflatoxin assay was carried out by the addition of 100 µL of each standard or sample into the appropriate dilution well contained 200 µL of conjugate using a single channel pipettor. Each dilution-well was mixed by carefully pipetting it up using an 8-channel pipettor with fresh tips. A total of 100 µL of the contents from each dilution well was immediately transferred into a corresponding antibody-coated microwell then incubated at room temperature for 15 min. After incubation, the contents of microwell strips were empty into a waste container. Each microwell washed with distilled or deionized water then dumped the water from the microwell strips and this step was repeated 4 times for a total of 5 washes. Several layers of tissue were put on a flat surface, and microwell strips were tapped on towels to expel as much residual water as possible after the fifth wash. The bottom of the microwells was dried with a tissue. A total of 100 µl of the substrate was added into each microwell strip using an 8-channel pipettor then incubated at room temperature for 5 min followed by the addition of 100 µl of stop solution
into each microwell strip using an 8-channel pipettor the color change from blue to yellow. The absorbance was read at 450 and 630 nm.

2.7. Nutrient composition
Nutrient content of USBM and FSBM, including moisture content, ash, crude fiber, crude protein, and lipid were performed in triplicate. Dry matter was measured at 105 °C until constant weight. Crude protein content was carried out by the Kjeldahl method based on AOAC [20] (Helrich, 1990). Crude fiber was determined as AOAC [20] (Helrich, 1990). Crude lipid was obtained by Soxhlet extraction, as described by Novakofski et al. [21].

2.8. Data analysis
One-way ANOVA followed by the Duncan Multiple Range Test (DMRT) comparison test was performed using Costat [22] to analyze the LAB population. Phytic acid, aflatoxin, and nutrient content were analyzed using a T-test to compare treatment means [23].

3. Result and discussion
3.1. Microbial population and growth curve
*Lactobacillus plantarum* is considered as a GRAS (Generally Regarded as Safe) and a genus *Lactobacillus* is a heterogeneous group of LAB with important implications in food fermentation. *Lactobacilli* are currently used as probiotics, silage inoculants, and as starters in fermented food [24]. Table 1 showed that the population of *L. plantarum* A1-E increased during fermentation time until 48 h (P<0.05) and stable between 48 and 72 h (P>0.05). Charalampopoulos et al. [25] reported that *L. plantarum* (0.20-0.41 log₁₀ CFU/ml) during 48 h fermentation attained the highest cell populations growing in malt medium (10.11 log₁₀ CFU/ml) compared to barley medium (9.43 log₁₀ CFU/ml) and wheat medium (9.29 log₁₀ CFU/ml).

| Fermentation time | Population CFU/ml | log₁₀ CFU/ml |
|-------------------|-------------------|--------------|
| 0                 | 2.1 x 10⁶ c       | 6.33±0.05 c  |
| 24                | 6.9 x 10⁶ b       | 6.80±0.22 b  |
| 48                | 6.4 x 10⁷ a       | 7.79±0.12 a  |
| 72                | 6.8 x 10⁷ a       | 7.82±0.11 a  |

SBM contained complex carbohydrates, proteins, vitamins, minerals, and fiber sources. Carbohydrates were converted by *L. plantarum* A1-E into simple sugars particularly glucose as the fermentation product. According to a prior study, natural fermentation increases glucose in soybean [26], thereby the growth of LAB was faster.

Log of number of viable bacteria in Table 1 then plotted in a graph against time by subtracting each data from 24, 48 and 72 h fermentation period with data from the time of inoculation of the bacteria (0-time) to obtain the growth curve (Figure 1). Growth of *L. plantarum* begins with an initial phase (lag) which is an adaptation phase, then followed by an exponential phase until 48 h incubation. Growth enters steady growth or a stationary phase at 48 to 72 h. Istiqomah [11] reported that the lag phase of *L. plantarum* A1-E on the modified MRS broth media at 37 °C incubation without agitation occurred in the first two hours and showed slow growth whereas the exponential phase was reached between the 2 – 10 h incubation and the stationary phase started at the 10 up to 24 h incubation, and afterward enters the death phase. Smetanková et al. [27] revealed that at 37 °C and 45 °C, the exponential phase of *L. plantarum* 2L2 was observed between 2-8 h during the transition to the stationary phase, there is a stimulation of the formation of some proteins and in the stationary phase, there is a lack of essential nutrients.
Figure 1. Growth curve of *L. plantarum* A1-E in SBM during fermentation.

The different exponential phase tends in this study compared to previous studies probably due to differences in a growth medium. SBM medium contains a greater amount of nutrients than MRS broth media thus *L. plantarum* A1-E cleaved carbohydrates in the media to be converted into simple sugars used for cell propagation so that cell growth continues to increase up to 48 h incubation. Charalampopoulos et al. [25] stated that *L. plantarum* grew exponentially until 10–12 h of fermentation and the viable cell densities declined slightly during the stationary phase (12–48 h).

Generally, LAB produced primary metabolites such as lactic acid, acetic acid, and hydrogen peroxide in the logarithmic phase and the stationary phase. In this phase, the synthesis of enzymes by bacterial cells is used for the metabolism of metabolites. After certain substrates or compounds needed for bacterial growth in the culture medium are nearing exhausted and there is a build-up of inhibitory products, there is a decrease in the rate of growth of LAB [28].

### 3.2. Phytic acid and aflatoxin content

Table 2 revealed that fermentation by *L. plantarum* A1-E (FSBM) during 72 h reduced 34.13% of phytic acid content (P<0.05) compared to control (USBM). *Lactobacillus plantarum* A1-E produced phytase with 41.9 U/ml of activity and 5.5 U/mg of specific activity [11]. Sofyan et al. [29] reported that fermentation of rice bran by *Rhizopus* sp. combined with deproteinated chitin waste (DCW) decreased phytic acid (35.1%). It has been reported that fermented millet grain reduced food inhibitors, phytic acid, and tannins [30]. Germinated pearl millet sprouts that fermented by mix pure cultures of *L. brevis*, *L. fermentum*, *Saccharomyces diasticus*, and *S. cerevisiae* at 30 °C for 72 h resulted in 88.3 % reduction in phytate content [31]. Natural fermentation can achieve a large reduction in anti-nutritional factors (phytic acid) in substrates by the action of microbial accompanied by a significant improvement in the protein digestibility. This producing phytase microbes were able to hydrolyze phytic acid in SBM into Myo-inositol (phosphate). This Myo-inositol (phosphate) then hydrolyzed by alkali phosphate in producing Myo-inositol and inorganic phosphorus (Pi). Hydrolysis of phytate during food processing and then preparation, for example by fermentation is a result of the phytate-degrading activity of phytase, which is naturally present in plants and microorganisms [3].

| Material | Phytic acid (g/100 g) | Aflatoxin (µg/kg) |
|----------|-----------------------|-------------------|
| USBM     | 1.38±0.11<sup>a</sup> | 3.08±0.11<sup>a</sup> |
| FSBM     | 0.91±0.07<sup>b</sup> | 2.89±0.17<sup>a</sup> |
Reduction (%) | 34.13 | 6.12
Differences superscript on the same column shown a real difference (P<0.05)

Aflatoxin content in SBM was 3.08 µg/kg. Al-Seeini [5] reported that the level of aflatoxin detected in feeding soybean was ranged from 1 to 14.8 ppb. Daga et al. [6] revealed that the aflatoxin level was low in natural SBM (0.5µg/kg). Nomura et al. [7] (2018) reported that the mean concentration of AFB1 in SBM was 1.1 µg/kg. FSBM during 72 h incubation reduced 6.12% aflatoxin content in SBM. Two L. plantarum strains Lb7 and Lb9 isolated from sourdough performed poorly AFB1 binding abilities only 2.1% and 5.2%, respectively [32]. Istiqomah et al. [33] reported that L. plantarum G1 addition in the aflatoxin-contaminated diet decreased the aflatoxin content (2.71 ppb) compared to control (9.01 ppb). Mechanism of aflatoxin binding ability in LAB due to the cluster on the LAB cell wall [34]. Moreover, the binding of aflatoxin by LAB appears extremely strain-specific and different binding compounds. The Lactobacillus genus (L. helveticus, L. brevis, L. acidophilus, and L. crispatus) has been found to have an S-layer made of protein subunits, while L. rhamnosus has polysaccharides (cell wall polysaccharide, peptidoglycan, and teichoic or lipoteichoic acids) where it binds aflatoxins [8].

3.3. Nutrient composition
The nutrient content of SBM (Table 3) reflected the differences in composition between SBM and FSBM. SBM contained ash 6.10%, crude protein 43.14%, crude fiber 4.56%, and crude lipid 5.43%. Banaszkiewicz [1] (2014) reported that SBM provides crude ash 6.65%, crude protein 44.40%, crude fiber 6.75%, and crude fat 2.18%. FSBM after 72 h fermentation had on average more protein (1.33-fold), total fiber (2.09-fold), and less lipid (0.71-fold) than USBM (P<0.05) whereas the crude fat content was kept at a stable level (P>0.05).

| Material | Moisture | Ash       | Crude protein | Crude fiber | Crude lipid |
|----------|----------|-----------|--------------|-------------|-------------|
| USBM     | 9.18±0.30b | 6.10±0.09b | 43.14±0.42b  | 4.56±0.07b  | 5.43±0.64a  |
| FSBM     | 32.72±0.27a | 6.91±0.21a | 57.48±0.43a  | 9.53±0.06a  | 3.87±0.93a  |

Enhancement (fold) | 3.56 | 1.13 | 1.33 | 2.09 | (0.71)
Differences superscript on the same column shown a real difference (P<0.05)

Chi and Cho [9] reported that FSBM by Bacillus amyloliquefaciens U304 could substantially improve both the nutritional quality and bioactivity of SBM. FSBM by S. cerevisiae improved nutritional quality (50% increase in fiber and 32%, 83% and 69% decreases in carbohydrate, lipid, and trypsin inhibitors, respectively) and increased antioxidant activity (up to 206%) [10]. Protein content in FSBM by B. subtilis or Aspergillus oryzae microbes was increased (8.37% and 0.34%, respectively) due to the degradation of allergens into peptides thereby peptides can be easily absorbed by an animal and transported within an organism [14]. Fermented rice bran by Rhizopus sp. combined with deproteinated chitin waste (DCW) decreased crude fiber contents (38.4%) [16]. Hydrolysis in SBM as a substrate during fermentation by L. plantarum A1-E probably due to its stronger proteolysis at 37 °C. Moreover, L. plantarum A1-E produced phytase that breaks the formation of phytate-protein and phytate-carbohydrate complexes making protein and carbohydrate more available and degradable thereby increased the nutritional value of SBM.

4. Conclusion
Producing phytase L. plantarum A1-E had an important application in human nutrition during fermentation of soybean meal (SBM). FSBM increased L. plantarum A1-E population (6.8x10^7 CFU/ml) during 48 h incubation, reduced 34.13% of phytic acid and 6.12 of aflatoxin content, and also resulted more crude protein (1.33-fold), crude fiber (2.09-fold), ash (1.13-fold), and less crude lipid.
(0.71-fold) than USBM. Therefore, this bio-modified SBM represents an improvement in nutritional quality and becomes potential raw material as a new protein source for food.

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