1. INTRODUCTION

1.1. Research Background

Based on Shrestha *et al.* (2010), the fermentation process is a proven method to improve flavor, texture, and nutritional quality of soybeans. Therefore, there are fermented foods from soybeans such as tempeh, soy sauce, miso, and natto. Natto is a traditional Japanese food product which is prepared by fermenting soybeans with *Bacillus subtilis*. Natto was covered with a white-colored viscous substance, sticky appearance, soft texture, and unique flavor (Wei *et al*., 2001). Soybeans are usually difficult to digest, but natto as probiotic food is highly digestible because protein molecules of the beans have been broken down by the bacteria during fermentation (Muramatsu *et al*., 2001). Fermented soybean using *B.subtilis* can increase antioxidant activity, anti-allergy activity and fibrinolytic function. Unfortunately, the metabolism *Bacillus* species released the ammonia odor that might affect the palatability of fermented food (Ying *et al*., 2009).

Lactic acid bacteria (LAB) are known as the best beneficial strains used to improve texture, aroma, flavor, and used in various ways such as bacteriocin producer, probiotic, and starter culture in fermented foods (Aktypis *et al*., 2007). Therefore, the addition of LAB may improve the palatability of fermented food produced by *Bacillus* species. Moreover, the whole soybeans fermented by using LAB mixed with *B.subtilis* natto in solid-state fermentation had received a potential biotechnological process because could provide different probiotics and had the capacity to improve functional and nutritional properties (Zhang *et al*., 2014).

Cowpea (*Vigna unguiculata* L. Walp) is a kind of legume which is cultivated across Southeast Asia, Africa, Southern Europe, Central and South America (Ratnaningsih *et al*., 2016). Based on Sayekti *et al.* (2010), cowpea has a potential production in Indonesia which reaches productivity around 1.5-2 ton/ha, depending on the varieties, locations, plant seasons, and cultivation ways. However, the major limiting factors of consumption cowpea for daily food are poor digestibility (Jayathilake *et al*., 2018). Usually, cowpea mostly consumed as cooked whole seeds after two hours of the boiling process resulting in high energy consumption and a long time for

preparation (Kayitesi *et al*., 2013). While the industrial processing and the utilization of cowpea was not being improved optimally and the beneficial effects of bioactive compounds in the cowpea also unexplored. The potential of cowpea needs to be processed into functional products would be influenced by their physical and chemical properties (Henshaw, 2008).

Soybean was replaced by cowpea in fermentation with *B.subtilis* and this final product will be called as cowpea natto. The whole cowpea fermented by *B.subtilis* natto mixed with LAB as probiotics carrier food was not investigated. To develop the bioactive compounds such as total phenolic content and antioxidant, the optimum fermentation conditions such as fermentation times and ratio of co-cultures need to determine by measuring characteristics of cowpea natto such as pH, color, antioxidant activity, and total phenolic content. Different fermentation time and different ratio of co-cultures *B.subtilis* and LAB was conducted to obtain a higher total phenolic content and antioxidant in produced natto from cowpea. During fermentation process, the antioxidants was increased due to the action of the microbial enzyme and hydrolysis of protein to amino acids and peptides (Zhang *et al*., 2014). Previous study has reported that red beans fermented with *B. subtilis* and *L. bulgaricus* had higher polyphenol and antioxidant properties than unfermented red beans (Jhan *et al*., 2015).

1.2. Literature Review

1.2.1. Cowpea (*Vigna unguiculata* L. Walp)

Cowpea (*Vigna unguiculata* L. Walp) or usually called as *kacang tolo* or *tunggak* (in Bahasa) was known as one of the most ancient food sources, and its seeds are the most economically valuable plant part of cowpea. Cowpea seeds contain almost the same nutritional as beans. Cowpea is considered as an excellent source of protein and health-promoting food such as soluble and insoluble dietary fiber, carbohydrate (glucose and fructose), phenolic compounds, minerals, vitamins of B complex, and a small quantity of lipids. The proximate compositions of cowpea range as follows: protein 17.4-31.7%; carbohydrate 35.7-65.7%; fat 1.00-3.03%; dietary fiber 19.5-35.6%; and mineral content 26-4.6% (Antova *et al*., 2013). Cowpea is rich in amino

acids such as valine, leucine, phenylalanine and lysine. The whole cowpea seeds contain phenolic acids, such as ferulic acid, cinnamic acid, caffeic acid, protocatechuic acid, 2,4-dimetoxybenzoic acid, and p-coumaric acid. Cowpea also has anti-nutrients such as trypsin inhibitor, hemaglutinin, and cyanogenic glycoside (Jayathilake *et al*., 2018). The seed coat pigments of cowpea contain 0.18-0.59% tannins and non-tannin phenolics (Siddhuraju and Becker, 2006).

Usually, cowpea seeds are consumed as boiled seeds and also can be processed into flour (Henshaw, 2008). Previous studies have shown that cowpea seeds are consumed as soup ingredients, traditional snacks, and can be utilized as cowpea tempeh or *tempe tolo* (Ratnaningsih *et al*., 2009). Sometimes, cowpea seeds also used as a coffee substitute (Siddhuraju and Becker, 2006). Some research has indicated that the consumption of cowpea provides protective effects for several chronic diseases, like cardiovascular diseases, hypercholesterolemia, obesity, diabetes, and several types of cancer (Ul-Haq *et al*., 2013).

Figure 1. Raw Cowpea (*Vigna unguiculata* L. Walp)

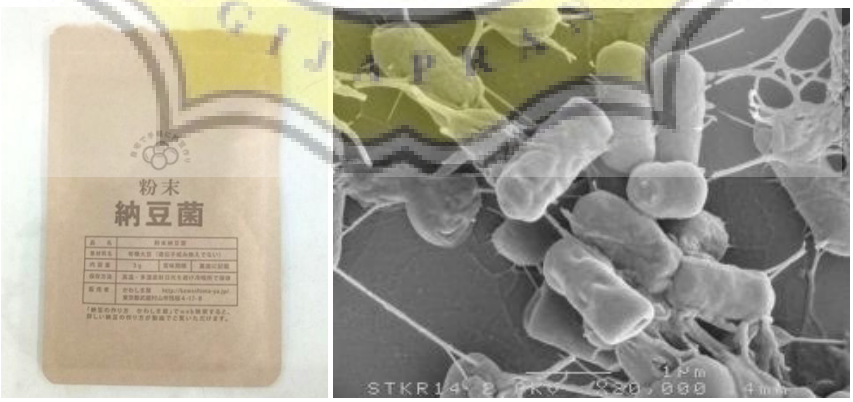
1.2.2. *Bacillus subtilis* var. natto

For fermented food products, they need some microorganism to help the process. Natto was commonly prepared by fermenting soybeans with *Bacillus subtilis*. *Bacillus* spp. is the most dominant natural fermenting agent in soybeans. *Bacillus subtilis* (natto) is a gram-positive, endospore-forming bacterium. The lack of

pathogenicity among *Bacillus* strains has resulted in Food and Drug Administration granted the organism as GRAS (Generally Regarded as Safe) (Shrestha *et al.*, 2010).

In previous research was shown that *Bacillus subtilis* var. natto could produce better quality natto in the formation of string, aroma, and flavor than *Bacillus subtilis* (Sulistyo *et al*., 1988). *Bacillus subtilis* var. natto was a bacterium isolated from natto that contains several proteases during soybeans fermentation (Hitosugi *et al.*, 2015). In this research, *Bacillus subtilis* var. natto from Japan was used as the starter. Parkouda *et al*. (2009) has explained that natto production was carried out industrially using starter cultures of *Bacillus subtilis* var. natto and now has already been commercialized all over the world.

B.subtilis (natto) grows on cooked beans and other foods such as cereals, meat, fish, shellfish, and dairy product. It utilizes glucose, sucrose, and fructose as carbon sources. Sucrose is important not only for growth but also for the production of viscous materials. *B.subtilis* (*natto*) also requires biotin (B vitamins) to grow on media and spore germination. In order to produce good quality natto, the optimum temperature for germination is about 40°C and grow at about neutral pH. Oxygen is required for the growth of the organism, it can grow at concentration of more than 3% oxygen (Steinkraus, 2004).



(source: microbewiki.kenyon.edu)

(a) (b)

Figure 2. *Bacillus subtilis* var. natto
"*Kawashima-ya*" starter (a), under the microscope (b)

1.2.3. Lactic Acid Bacteria (LAB)

Lactic Acid Bacteria (LAB) are gram-positive, bacilli and cocci shapes, produce lactic acid as the main product, and well known for its GRAS status. LAB has a long history of application to their advantageous influence on nutritional values, shelf-life, and organoleptic characteristics of the fermented foods. LAB are well known to be major beneficial microorganism in human intestine and have been widely utilized to manufacture fermented soy products such as soy flour, soymilk, sufu (a Chinese fermented soybean food) (Zhang *et al*., 2014).

In previous study was found the method to eliminate the ammoniacal odor of natto and it would be beneficial health in the human intestinal bacterial flora. It was natto contains lactic acid bacteria or referred to "*Yogurunatto*", which involves growing *Bacillus* natto and lactic acid bacteria on steamed beans with the presence of a nutritive medium (Naruse and Naruse, 1987). Hosoi *et al*. (2000) has reported that *B. subtilis* natto enhances the growth and viability of lactobacilli. During the incubation period of *B subtilis* natto and lactobacilli, the protein and peptide content of medium has changed as the enhancement effect.

Lactic acid bacteria used in this research was *Lactobacillus delbrueckii* KSM 10 from a marinade of tempeh making. *Lactobacillus delbrueckii* ssp. *delbrueckii* is an isolate that was identified at day 2 of tempeh production based on fermentation of carbohydrate concerning sugar utilization and acid production of each species (Pisol *et al*., 2015). *Lactobacillus delbrueckii* is a gram-positive bacterium, non-motile, facultatively anaerobic, non-spore-forming, and acid tolerant. It generally grows at the range of temperature 30-40°C (Tortora *et al*., 2013).

1.2.4. Fermentation Process of Natto

Fermentation usually involves microorganisms such as lactic acid bacteria, yeasts, moulds, and some species of bacteria (Shrestha *et al.*, 2010). Natto is one of traditional Japanese food products made by fermenting the beans with *Bacillus subtilis*. Based on the history of Natto (Shurtleff and Aoyagi, 2012), natto is a type of food made from boiled grains or seeds which is put into an incubation chamber.

Concerning the origin of the word "natto" and according to the theory, "*na"* is originated from *nasso*, which refers to a temple's kitchen and "*to"* means 'beans'.

The processing of natto includes soaking, steaming, cooling, inoculating, fermenting, and aging (Weng and Chen, 2010). Based on the method of Wei *et al*. (2001) and Jhan *et al*. (2015), the beans are first soaked in water for 16 hours at the room temperature and cooked under steam pressure 121°C for 1 hour. The cooked beans were cooled (30-40°C) and were inoculated quickly with *B.subtilis* var. natto. Natto formation usually appears at 14-18 hours of fermentation at 35-40°C. Before natto is ready to serve, natto was put in the chiller or refrigerator (± 4°C) 24 hours for the ripening process. Natto has a high moisture content of 59.5%. Cold storage has been used to extend its shelf life. Natto food must be usually chilled lower than 10°C for storage condition (Weng and Chen, 2010).

Depends on the final product, fermentation of natto was categorized as alkaline fermentation. Alkaline fermentation was defined as a fermentation process which the pH of the substrate increases to alkaline values as high as pH 9 due to degradation of proteins from the raw material into peptides, amino acids, and ammonia (Parkouda *et al*., 2009). Moreover, the protease of *B. subtilis* natto is subtilisin and endoprotease (Steinkraus, 2014). Alkaline products were known to enhance the nutritional value and shelf life of food (about a week), provide a variety of flavors, and decrease the natural toxin content in raw materials (Wang and Fung, 1996).

1.2.5. Characteristics of Natto

Good quality of natto products was covered with a white-colored viscous substance and had a sticky appearance when mixed or stirred with chopsticks, soft texture, and unique flavor (Wei *et al.*, 2001). Based on Kada *et al*. (2008), sticky formation during fermentation of natto is a high-quality product characteristic. When natto was stirred, the viscous and sticky texture depends on the γ-polyglutamic acid (γ-PGA) that forms on the surface of beans. Poly-glutamic acid (PGA) is an amino acid polymer and it produced from *B. subtilis.* The viscous materials of natto is safe to eat because it is biodegradable, water-soluble and non-toxic for human, it is one of the

functional properties of microorganism (Chettri *et al*., 2016). During fermentation, ammonia will be produced so, natto has an off-flavor characteristic involving an ammoniacal odor, which increases along with its storage time (Zhang *et al*., 2014).

Most people eat natto by mixing it in a bowl with condiments, egg, soy sauce, and mustard then spreading it over hot rice. Natto also can be eaten in the form of natto soup. Natto can be used as a flavoring agent for the preparation of vegetable dishes, meat, and seafood, and as the ingredient for sauce production (Wei *et al*., 2001). Natto can be used as a health supplement because of its high amount of nutrients: 100 g of natto contains a variety amino acid (16 g), vitamin B1 (0.07 mg), vitamin B2 (0.56 mg), vitamin B6 (0.26 mg), and fibers (6.7 g) (Hitosugi *et al.*, 2015). Traditionally, people used natto to treat vascular and heart diseases, as an antiberiberi agent, and to relieve fatigue. Natto intake also reported to support bone formation in menopausal women and to prevent the bone loss of postmenopausal due to the presence of menaquinone or isoflavones in natto (Parkouda *et al*., 2009).

1.3. Objective

The objective of this research was to know the effect of fermentation time and ratio of co-cultures *Bacillus subtilis* var. natto and *Lactobacillus delbrueckii* KSM 10 on the physicochemical properties such as pH, color, antioxidant activity, and total phenolic content, and also the sensory properties of cowpea natto.