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# Antibacterial Activity of the Purified Monolaurin from Enzymatic Glycerolysis of Palm Kernel Olein-Stearin against *Bacillus subtilis*

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## Abstract

**Background and Objective:** Monolaurin can be produced by enzymatic glycerolysis of a palm kernel olein-stearin mixture. Monolaurin can act as an antibacterial agent against Gram-positive and Gram-negative bacteria. Few studies have investigated activity of monolaurin against *Bacillus subtilis* cells and spores. Therefore, the aim of this study was to investigate effects of a purified monolaurin from enzymatic glycerolysis of palm kernel olein-stearin mixture on growth, spore germination, cell surface hydrophobicity and cell structure of *Bacillus subtilis* FNCC 0060.

**Material and Methods:** Monolaurin was produced using palm kernel-olein mixture under the best enzymatic glycerolysis condition and purified using solvent (hydroalcoholic) method. Effects of a purified monolaurin on *Bacillus subtilis* growth and spore germination were studied using nutrient broth. Analysis of cell surface hydrophobicity was carried out using microbial adhesion to hydrocarbons assay and cell structure was studied using transmission electron microscope. Completely randomized experimental design was used and each treatment was carried out in triplicate. Analysis of variance and Duncan multiple range tests were used to analyze data.

**Results and Conclusion:** Results showed that purified monolaurin inhibited growth of *Bacillus subtilis* FNCC 0060. Moreover, development of *Bacillus subtilis* in nutrient broth with monolaurin of 100-5,000 $\mu g.ml^{-1}$ was slower than that without monolaurin. Higher concentration in the media decreased the microbial specific growth rate and increased doubling time. Spores of *Bacillus subtilis* FNCC 0060 in nutrient broth with monolaurin of 100-1,000 $\mu g.ml^{-1}$ delayed germination for up to seven days of incubation. Presence of monolaurin significantly decreased bacterial cell adherence. Furthermore, cytoplasm of *Bacillus subtilis* FNCC 0060 seemed shrunk significantly, causing cytoplasmic damages and disorganization of the components.

**Conflict of interest:** The authors declare no conflict of interest.

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## 1. Introduction

Foodborne diseases are global concerns that are increasing rapidly and include detrimental consequences for food supply and human wellness [1]. Foodborne pathogens cause various illnesses. Therefore, food industries concern about ensuring that their products are microbiologically safe. Food additives are used to prevent bacterial and fungal contaminations [2]. To extend shelf-life of foods and prevent microorganism growth, various types of antimicrobial agents are used as food components or packaging materials [2,3]. Antibiotics have become standard treatments for the bacterial infections due to their great potency and ability to target a broad spectrum of pathogens. These chemicals are commonly used as protective measures in treatment of suspected infections [4]. Natural antimicrobial compounds and preservatives are preferred by the consumers over synthetic chemicals [2,5]. To address this problem, lipids such as monoglycerides seem to be promising candidates for antibacterial agents to treat bacterial infections [4]. Monoacylglycerols are widely used in cosmetic and food industries as emulsifiers and preservatives [6-8]. Monoglycerides include antimicrobial characteristics against microorganisms of various types, including Gram-negative and Gram-positive bacteria [9]. Several studies have been carried out in recent years on the antibacterial characteristics of fatty acids as well as their monoglycerides such as lauric acid and monolaurin [5,10]. Monoglycerides in the form of monolaurin and monocaprin include greater antibacterial activities than those the free fatty acids such as lauric acid and capric acid do [11]. The Food and Drug Administration has classified monolaurin or glycerol monolaurate (GML) as a recognized, safe natural compound with strong antibacterial activity against Gram-positive cocci and *Bacillus* (*B.*) *anthracis* [12].

Palm kernel oil (PKO) can be used to produce monolaurin. The PKO is a byproduct of the crude palm oil processing and has increased productivity. Moreover, PKO is a feasible monolaurin feedstock that can be separated into palm kernel olein (PKOo) and palm kernel stearin (PKS). A previous study detected that a 40:60 (w.w$^{-1}$) PKOo-PKS (Palm kernel olein-stearin) mixture produced 24.58% ±0.56 of glycerol trilaurate and 57.01% ±0.15 of lauric acid values. The PKOo-PKS proportion of 40:60 (w.w$^{-1}$) contains lauric acid at the sn-2 position of 50.71% [13]. Furthermore, a PKOo:PKS ratio of 40:60 achieved monolaurin yields of 2.18% ±0.59 and 3.47% ±0.62 after 3 and 24 h, respectively [14]. Ngatirah et al. reported that the best glycerolysis requirements for the synthesis of monolaurin were achieved with 10% w.w$^{-1}$ enzyme concentration, oil-glycerol molar ratio of 1:4, solvent-to-oil ratio of 2:1 v.w$^{-1}$ and glycerolysis temperature of 40 °C at 600 rpm [15]. Monocaprin and monolaurin included greater antibacterial activities against Gram-positive bacteria such as *B. Staphylococcus* and *Enterococcus* than Gram-negative bacteria such as *Escherichia* (*E.*) *coli, Klebsiella, Pseudomonas* and *Acinetobacter* [5,15,16]. Several Gram-positive strains are inhibited by monocaprin, monolaurin and monomyristin, with monolaurin including the highest inhibitory activity [17,18].

Limited studies have investigated antibacterial activity of monolaurin on *B. subtilis* cells and spores. Naturally, *B. subtilis*, *B. stearothermophilus* and *B. amyloliquefaciens* are the most common bacteria in *Bacillus* genus that cause degradation of processed foods [19]. The *B. subtilis* is a bacterium that is not pathogenic; however, its spores include the potential to contaminate environmental surfaces such as workbenches of laboratories and ventilation systems. Previous studies have reported that monolaurin includes antibacterial effects when dissolved in ethanol [5,12]. Inhibition zone of microemulsion monolaurin against *B. subtilis* was greater than that against *Escherichia coli* and *Staphylococcus* (*S.*) *aureus* [20]. According to Zhang et al., minimum inhibitory concentrations of monolaurin against *E. coli*, *S. aureus* and *B. subtilis* were 25, 12.5 and 30 g.ml$^{-1}$, respectively [21]. Schlievert et al. detected that monolaurin or GML effectively killed vegetative cells of *B. subtilis*, *B. anthracis*, *Clostridium* (*C.*) *perfringens* and *C. difficile*. Furthermore, GML solubilized in a nonaqueous gel effectively killed *Bacillus* and *Clostridium* spores [12]. The *B. subtilis* growth was inhibited more strongly by microemulsion monolaurin [22]. Furthermore, monolaurin included greater effects on *B. subtilis* than other bacteria (e.g., *S. aureus*, *Pseudomonas* (*P.*) *aeruginosa* and *E. coli*) and minimum inhibition concentration against *B. subtilis* was approximately 0.63 mg.ml$^{-1}$ [5]. Presence of mono and diglycerol FA esters significantly decreased viable spore counts of *B. subtilis* [23]. The study detected that synergistic nisin and monolaurin affected heat-resistant bacterial spores. The optimum process parameters for the maximum bacterial spore decrease (~3 log) were achieved at nisin concentrations greater than 150 IU.ml$^{-1}$ and monolaurin concentrations greater than 200 µg.ml$^{-1}$ [24]. However, there is a little information on the antibacterial activity of monolaurin by enzymatic glycerolysis of a PKOo-PKS mixture dissolved in water on *B. subtilis*, including its effects on growth and spore germination and mechanisms of action. The aim of this study was to investigate effects of monolaurin derived from the enzymatic glycerolysis of a PKOo-PKS mixture on growth and spore germination, cell surface hydrophobicity and cell structure of *B. subtilis* FNCC 0060.

## 2. Materials and Methods

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### 2.1. Sample collection

Samples were collected from growth media (with or without monolaurin) every 2 h until 24 h. Samples were serially diluted to calculate number of bacteria using plate count method. To assess effects of monolaurin on spore germination, 5-ml samples were collected every day for seven days and measured spectrophotometrically at 625 nm to record optical density values. The biomass cells were collected from nutrient broth media with and without monolaurin at 100 µg.ml$^{-1}$ concentration for preparing TEM (transmission electron microscope) micrographs. Biomass cells were collected by centrifuging at 3000 rpm for 15 min.

### 2.2. Fat mixture preparation

Fat mixture was prepared based on a procedure by Ngatirah et al. [13]. Separately, refined RBD-PKOo and RBD-PKS were melted at 70 °C for 30 min to homogenize the oil fractions. Then, melted RBD-PKOo and RBD-PKS were mixed at 40:60 (w.w$^{-1}$) ratio, stirred until homogeneous at 70 °C for 15 min and stored in refrigerator until use.

## 2.3. Monolaurin production and separation

Monolaurin was produced under the optimal enzymatic glycerolysis condition, as described in a previous study [15]. A mixture of RBD-PKOo and RBD-PKS (PT Wilmar, Jakarta, Indonesia) at a 40:60 w.w$^{-1}$ ratio of 100 g was melted at 70 °C for 30 min and mixed with glycerol (PT Wilmar, Jakarta, Indonesia) at a 1:4 molar ratio. *Tert*-butanol mixture (Merck KGaA, Darmstadt, Germany) at a 2: 1 oil-to-*tert*-butanol ratio was added to the current mixture and molecular sieve (Sigma-Aldrich. St. Louis, MO, USA) 12% w.w$^{-1}$ of glycerol was added to mixture. At 40 °C, the mixture was transferred into a batch-stirred tank reactor and mixed well at 600 rpm. Lipozyme RM IM (Sigma-Aldrich, St. Louis, MO, USA) was added to the mixture with 10% w.w$^{-1}$ oil (300 U.g$^{-1}$) and then incubated at 40 °C for 24 h. Centrifugation (Hettich EBA 200, Germany) was carried out at 3000 g for 5 min to separate product from the enzyme and glycerol. Rotary evaporator (Heidolph, Germany) was used to separate product from the solvent. Monolaurin separation was carried out based on a method described by Nitbani et al. [25] with modifications. Hydroalcoholic solvent (ethanol:water of 8:2) was used to separate monolaurin at a 1:9 v.v$^{-1}$ ratio and chilled for 24 h. Diacylglycerol (DAG) and triacylglycerol were crystallized while MAG was dissolved in the hydroalcoholic phase. Then, these were vacuum-filtered. The filtrate (containing monolaurin and solvent) was mixed with *n*-hexane (Merck KGaA, Darmstadt, Germany) in a 1:3 v.v$^{-1}$ ratio and set for 24 h to form two layers using separating funnel. The bottom layer was collected to achieve monolaurin and then hydroalcoholic was removed using rotary evaporator (Heidolph, Germany) [25]. The purified product was stored in refrigerator. Purified monolaurin was further identified using fourier transform infra-red (FTIR), nuclear magnetic resonance and gas chromatography, previously published by Ngatirah et al. [15]. Purified monolaurin was used to assess its effectiveness as an antibacterial agent on *B. subtilis* (Food and Nutrition Culture Collection/FNCC, Universitas Gadjah Mada's Center for Food and Nutrition Studies, Yogyakarta, Indonesia), which included effects on cell growth, spore germination, cell hydrophobicity and cell structure *B. subtilis.*

## 2.4. Effects of monolaurin on growth of *Bacillus subtilis* FNCC 0060

Effects of monolaurin on *B. subtilis* FNCC 0060 was assessed based on a protocol by Hauerlandova [26] with modifications. Nutrient broth (100 ml) (Merck KGaA, Darmstadt, Germany) was prepared using lidded flasks before adding monolaurin at final concentrations of 0, 100, 500, 1,000, 2,500 and 5,000 µg.mL$^{-1}$. Furthermore, pH of the media was set at 6.8-7 and sterilized at 121 °C for 15 min. Each treatment was inoculated with 0.1-ml suspension of *B. subtilis* FNCC 0060 (approximately $10^5$-$10^6$ cell.ml$^{-1}$) and then incubated at 37 °C for 24 h. Every 2 h, a sample was collected to analyze the number of bacteria using plate count method. Number of the cells (CFU.ml$^{-1}$) was plotted against incubation time to achieve a growth curve. Specific growth rate (µ) and doubling time of the microorganisms were calculated using growth curve in the logarithmic phase. Slope of the linear part in the exponential phase showed the specific growth rate (µ) value. Doubling time was calculated using Eq (1):

$$\text{Doubling time (dt)} = \frac{\ln(2)}{\mu} = \frac{0.693}{\mu} \qquad \text{Eq. 1}$$

## 2.5. Effects of monolaurin on spores of *Bacillus subtilis* FNCC 0060

Effects of monolaurin on spores of *B. subtilis* FNCC 0060 were assessed based on a protocol by Hauerlandova [26] with modifications. *Bacillus subtilis* cultures were prepared in nutrient broth (Merck KGaA, Darmstadt, Germany) and incubated at 30 °C using orbital shaker (50 rpm). After incubation, 1 ml of the cell suspension was poured onto the nutrient agar (Merck KGaA, Darmstadt, Germany) surface and incubated at 30 °C until sporulation occurred. After microscopic analysis, free endospores were collected from the plate surface by washing five times with sterile distilled water. Spore suspension was centrifuged at 10,000 × g for 15 min at 4 °C and resuspended in 10 ml of sterile distilled water. The suspension spores were heated at 80 °C for 10 min and stored in refrigerator for the spore germination assay. Purified monolaurin was added to the nutrient broth to achieve concentrations of 100, 500 and 1,000 µG.Ml$^{-1}$. Nutrient broth (Merck KGaA, Darmstadt, Germany) without this compound was prepared as control and all conditioned media were sterilized at 121 °C for 15 min. Then, 100 µl of the spore suspension were inoculated into the nutrient broth (Merck KGaA, Darmstadt, Germany) and incubated at 37 °C for 7 d. Every day, growth was assessed spectrophotometrically at 625 nm. To prepare growth curves, optical density (OD) values were plotted against time and the inhibition index (II) [27] was calculated using Eq. 2:

$$\text{Inhibition index} = 1 - \frac{(change\ in\ OD625\ of\ the\ experimental\ culture)}{(change\ in\ OD625\ ofthe\ control\ culture)} \qquad \text{Eq. 2}$$

## 2.6. Analysis of the cell surface hydrophobicity

Microbial adhesion to hydrocarbons (MATHS) assay was used to assess CSH based on a protocol by Dufour et al. with modifications [28]. The *B. subtilis* FNCC 0060 cells were cultured in nutrient broth (Merck KGaA, Darmstadt, Germany) with monolaurin at concentrations of 0 and 100 µg.ml$^{-1}$ and incubated at 37 °C for 24 h followed by centrifugation at 3,500 rpm for 10 min. Supernatant was discarded and cell biomass was thoroughly washed with ice water and resuspended in 5 ml of phosphate buffer solution (absorbance at $\lambda_{500}$ reached 0.4). Moreover, 4.8 ml of the suspension of bacteria were added 0.8 ml of *n*-hexadecane (Merck KGaA, Darmstadt, Germany), vortexed for 1 min and then set for 30 min before measuring OD by spectrophotometer (Shimadzu, Japan) at $\lambda_{500}$. The cell surface hydrophobicity was calculated by bacterial affinity to solvents and expressed as adherence proportion using Eq. 3:

$$\text{Adherence } (\%) = (1 - A / Ao) \times 100\% \qquad \text{Eq.3}$$

Where, Ao was OD of the bacterial cell suspension before mixing with *n*-hexadecane and A was OD of the bacterial cell suspension after mixing with *n*-hexadecane.

### 2.7. Cell analysis using transmission electron microscope

Briefly, TEM analysis was carried out based on a method by Dufour et al. with modifications [28]. The *B. subtilis* FNCC 0060 cells were cultured in nutrient broth with 100 $\mu g.ml^{-1}$ monolaurin and without this compound for 24 h. Bacterial cells were centrifuged at 3,000 rpm for 15 min, supernatant was discarded and the biomass cells were rinsed with 1 ml of cacodylate buffer, stirred and rinsed three times. Then, 1 ml of fixative solution (2.5% glutaraldehyde) was added to the biomass cells and stirred for 15 min at 4°C. Cell analysis was carried out using TEM (TEM Jeol 1010, 80.0 KV, 30,000×).

### 2.8. Data analysis

Completely randomized design was used and each treatment was carried out in triplicate. One way analysis of variance and SPSS software v.20 were used to analyze data. Differences were analyzed using Duncan's multiple range tests (DMRT). Probability values less than 0.05 were considered statistically significant. Results were reported as means and standard deviations (SD).

## 3. Results and Discussion

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### 3.1. Effects of monolaurin on growth of *Bacillus subtilis* FNCC 0060

Growth curves of *B. subtilis* FNCC 0060 in nutrient broth media with the addition of monolaurin at various concentrations are seen in Figure 1. The *B. subtilis* is a Gram-positive, rod-shaped nonpathogenic bacteria that can produce spores. Figure 1a shows that the initial concentration inoculated into the nutrient broth was nearly 5.91 logs $CFU.ml^{-1}$. The highest growth rate occurred around the Hour 10 with the peak of the logarithmic phase with 8.01 log $CFU.ml^{-1}$ cells for control (without monolaurin). Addition of monolaurin at concentrations of 100-5,000 $\mu g.ml^{-1}$ caused a longer lag phase (nearly 4 h). This result was similar to that of Bunkova et al., where lag phase of *B. subtilis* growth curve on the control (without monolaurin) ranged 1.58 h ±0.17 with an extension of the lag phase when monolaurin was added into the growth media [17]. Figure 1a demonstrates that the growth curve of *B. Subtilis* in nutrient broth without monolaurin (0 $\mu g.ml^{-1}$) included a short lag phase and reached the peak at Hour 8, while the stationary phase occurred after incubation of more than 8 h.

This result was similar to that by Andriani et al., where *B. subtilis* cultured in nutrient broth included a short lag phase at Hours 4-8 of incubation [29]. Growth in Luria-Bertani (LB) media at 37 °C reached the stationary phase after Hour 8 [30]. Figure 1a shows that the growth curve in nutrient broth with monolaurin at concentrations of 100-5,000 $\mu g.ml^{-1}$ included a lag phase of nearly 4 h, then reaching the logarithmic phase after 4 h of incubation until Hour 16 followed by the stationary phase. These results demonstrated that monolaurin affected growth of the bacterial cells. Schlievert et al. reported that adding 100 $\mu g.ml^{-1}$ monolaurin was bactericidal to *B. subtilis* cultured in Todd-Hewitt broth but bacteriostatic at 50 $\mu g.ml^{-1}$ [12]. Monolaurin (250 $\mu g.ml^{-1}$) included a long-lasting bacteriostatic effects followed by a continuously lower regrowth rate than the control culture of *B. cereus* ATCC 14579T [31]. Wang et al. reported that monolaurin included antibacterial activities against *B. subtilis* in nutrient broth at a concentration of 0.63 $mg.ml^{-1}$ (630 $\mu g.ml^{-1}$) [5]. Within the first 8 h, this compound demonstrated a greater antibacterial activity against *S. aureus* than that pulegone did. However, no significant differences were reported in their effects after 24 h of incubation [1]. The number of cells of *B. subtilis* in the control (nutrient broth without monolaurin) was higher than that in nutrient broth with monolaurin. Therefore, inhibition of growth occurred possibly because of the presence of monolaurin in the media. The smallest number of cells was detected in *B. subtilis* cultured in nutrient broth with monolaurin at 5,000 $\mu g.ml^{-1}$.

Colony log differences of *B. subtilis* FNCC 0060 between the control and treatment are shown in Figure 1b.

These were higher in the logarithmic phase (4-8 h incubation) than others because cells were easily affected by the presence of antibacterial compounds. Differences in the number of colonies in the control and monolaurin-treated groups revealed decreases in cell counts. At Hour 8 of incubation, more than 2 log cycles were reported in development of the cells at monolaurin doses of 1,000-5,000 $\mu g.ml^{-1}$. After reaching the stationary phase, number of the cells decreased by less than 1 log cycle. Zhang et al. detected decreases in 2 log cycles in *E. coli* cells cultured on TSB media by adding monolaurin at 500 $\mu g.ml^{-1}$. In *S. aureus*, decreases in cell count at concentrations of 100-2,500 $\mu g.ml^{-1}$ were relatively similar at 0.11-0.16 log $CFU.h^{-1}$ and dropped significantly at 5,000 $\mu g.ml^{-1}$ around 0.27 log $CFU.h^{-1}$ [32]. Schlievert et al. reported that adding monolaurin to cow milk with concentrations of 3,000 and 5,000 $\mu g.ml^{-1}$ included bactericidal activity [33]. Specific growth rate and doubling time of *B. subtilis* FNCC 0060 in nutrient broth with monolaurin addition at various concentrations are shown in Figure 2.

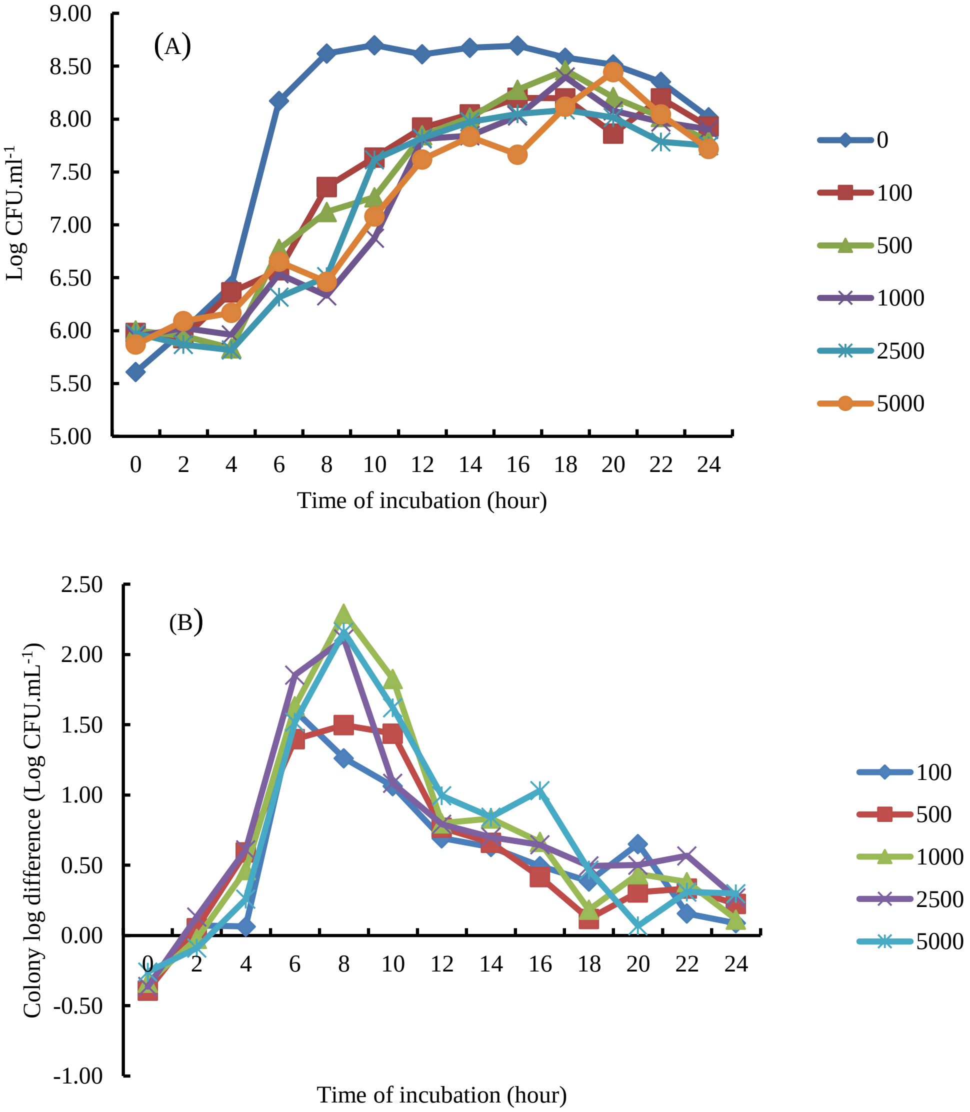

**Figure 1.** Effects of monolaurin on *Bacillus subtilis* FNCC 0060 growth in nutrient broth with monolaurin at various concentrations, µg.ml$^{-1}$ (A) and colony log difference of the bacteria between the control (without monolaurin) and treatment with monolaurin, µg.ml$^{-1}$ (B)

Figure 2 indicates that the specific growth rate decreased with increases in concentration of monolaurin in growth media. In the control, specific growth rate reached 1.65 per hour. This result was higher as Van Heerden et al. reported a growth rate of *B. subtilis* of 0.61 per hour with a generation time of 70 min [34]. Addition of monolaurin at 100 ppm decreased the growth rate to 0.96 per hour with a doubling time of 43.40 min. Further decreases in the growth rate occurred when the concentration was 5,000 µg.ml$^{-1}$ with a growth rate of 0.78 per hour and a doubling time of 53.36 min. This finding was similar to finding of Bunkova et al. that adding monoacylglycerol to Gram-positive bacteria resulted in a lower specific growth rate and increased concentration while decreased the maximum specific growth speed (µmax) [17]. Figure 2 demonstrates that monolaurin concentration in media was inversely proportional to the doubling time. In the control (without monolaurin), *B. subtilis* included a doubling time of 25.23 min. This result was similar to that of Budianto and Suprastyani, who reported a doubling time of 30.62 min for *B. subtilis* [35]. In the present study, *B. subtilis* in the control included cell division every 25.23 min. At monolaurin presence, cell division of *B. subtilis* was longer. At 100 and 5,000 µg.ml$^{-1}$ concentrations, doubling times were 43.40 and 53.36 min, respectively. This showed that adding monolaurin could inhibit growth of *B. subtilis*. Inhibition occurred because monolaurin damaged the bacterial cell membrane.

Increased cell growth and concentration of monolaurin decreased the specific growth rate and increased the doubling time of *B. subtilis* (Figure 2). This was possibly due to monolaurin as a relatively small nonionic surfactant with a spherical shape of nearly 0.95 nm in diameter. Therefore, monolaurin molecules could insert through the peptidoglycan and then interacted with the membrane through electrostatic and hydrophobic interactions. Hence, it arrested the cell cycle and ultimately interrupted the cell division. Zhang et al., (2018) reported that monoglycerides first crossed the *E. coli* membrane and then affected genomic DNA and damaged its double helix, affecting RNA synthesis and subsequent synthesis of proteins and DNA. Thus, this ultimately led to termination of the cell cycle and ultimately resulted in interruption of the cell division [32].

### 3.2. Effects of monolaurin on spore germination of *Bacillus subtilis* FNCC 0060

Effects of monolaurin on *B. subtilis* spore germination are shown in Figure 3.

Figure 3 shows delayed spore germination of *B. subtilis* FNCC 0060 in nutrient broth with monolaurin at 100-1,000 µg.ml$^{-1}$. In the control (0 µg.ml$^{-1}$), germination of *B. subtilis* spores occurred within 24 h with an OD of 0.7. At a monolaurin concentration of 100-1,000 µg.ml$^{-1}$, germination was still slow with OD reaching nearly 0.2. On Days 3-7, germination of *B. subtilis* spores increased. The slowest germination and growth rates occurred in nutrient broth media with addition of 1,000 ppm of monolaurin. This indicated that monolaurin could inhibit spore germination of *B. subtilis*. Results were similar to results by Klangpetch et al., who reported that FA esters (monolaurin and monocaprin) included inhibitory effects on the growth of *B. subtilis* spores [23]. Green et al. investigated that GML in a non-aqueous gel (GMLg) was ineffective as a sporicide; however, it could prevent germination of spores or kill germinated spores. [36]. A combination of nisin and monolaurin could inactivate *Bacillus* spores [36]. The optimum process for the maximal decreases in bacterial spores (3 log cycles) was recorded at concentrations of niacin greater than 150 IU.ml$^{-1}$ and monolaurin greater than 200 µg.ml$^{-1}$ [24]. Monolaurin in form of gel with a concentration of 5% (50,000 µg.ml$^{-1}$) could kill spores of *B. subtilis* after 1 h of inoculation [12]. The maximum decreases in *B. sporothermodurans* were reported at acidic pH and concentrations of 250–300 µg.ml$^{-1}$ [24]. The II of monolaurin to spore germination of *B. subtilis* is shown in Table 1.

The II of monolaurin against the spore germination of *B. subtilis* in nutrient broth during 24-h incubation at concentrations of 100, 500 and 1,000 ppm reached 0.71-0.74. Longer incubation time resulted in decreases in monolaurin II to spore germination. On Day 7 of incubation, II of monolaurin against the germination of *B. subtilis* spores reached 0.21, 0.30 and 0.32 for 100 ppm, 500 ppm and 1,000 ppm, respectively.

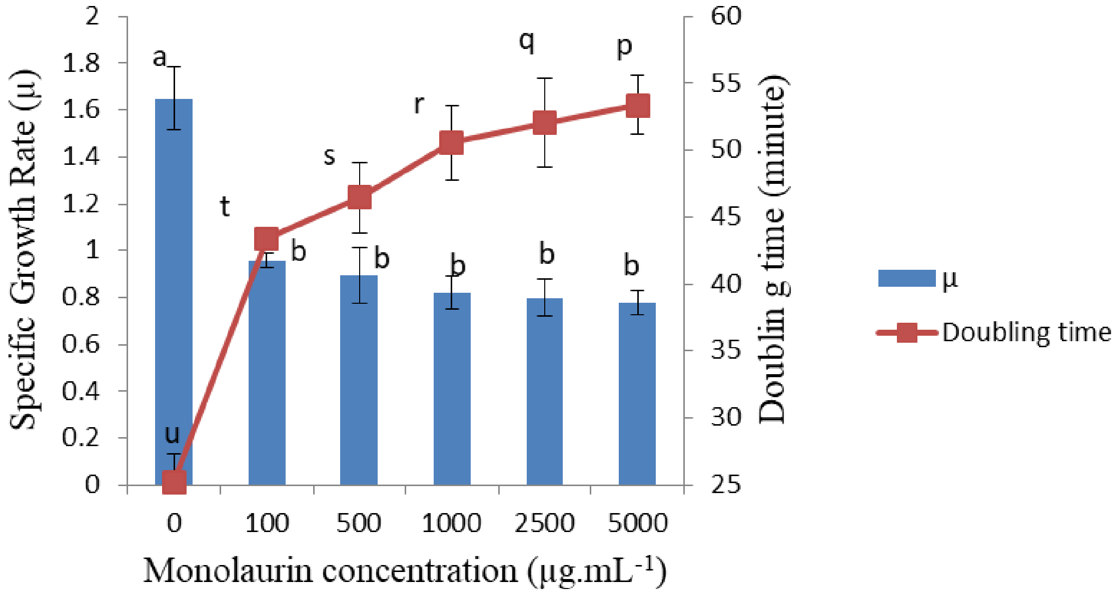

**Figure 2.** Specific growth rate and doubling time of *Bacillus subtilis* FNCC 0060 in nutrient broth with monolaurin addition at various concentrations

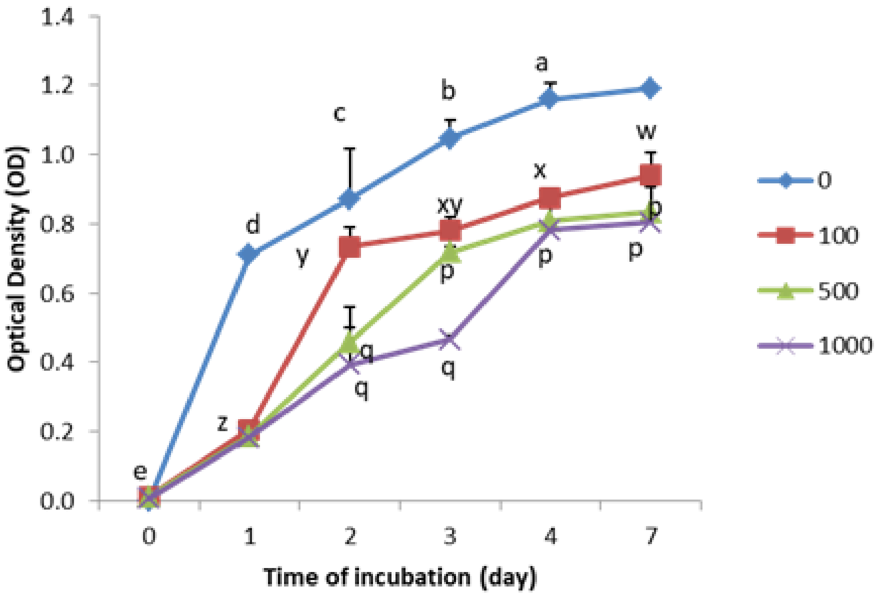

**Figure 3.** Spore germination of *Bacillus subtilis* FNCC 0060 in nutrient broth with addition of monolaurin at various concentrations (µg.ml$^{-1}$)

**Table 1.** Inhibition index of monolaurin to germination of *Bacillus subtilis* spores

| Monolaurin concentration (µg.ml$^{-1}$) | Time incubation (Days)* 1 | 2 | 3 | 4 | 7 |
|---|---|---|---|---|---|
| 100 | 0.71±0.01$^{a}$ | 0.16±0.08$^{d}$ | 0.25±0.00$^{d}$ | 0.25±0.02$^{d}$ | 0.21±0.06$^{d}$ |
| 500 | 0.74±0.03$^{a}$ | 0.47±0.21$^{b}$ | 0.32±0.05$^{c}$ | 0.30±0.06$^{c}$ | 0.30±0.12$^{c}$ |
| 1000 | 0.74±0.01$^{a}$ | 0.55±0.05$^{b}$ | 0.56±0.04$^{b}$ | 0.33±0.07$^{c}$ | 0.32±0.08$^{c}$ |

*value is average ± SD (n = 2). A row's average, followed by a different letter, indicates a significant difference in the Duncan Multiple Range Test (DMRT) 5%

Interactions were seen between the monolaurin concentration and time incubation. Increasing the concentration increased the II. However, longer incubation times decreased the II. Longer incubation times triggered germination and growth of spores into vegetative cells due to the presence of agents that triggered spore germination, including nutrients with specific amino acids (AAs) such as alanine, valine and asparagine [37].

The II on Day 7 was still greater than 0. It revealed that monolaurin still inhibited spore germination until Day 7 of incubation. The II of 0 and 1 indicated no inhibitions at all. The II value of smaller than zero (II value<0) indicated vegetative cell multiplication greater than the control, while a value of II of greater than one (II value>1) indicated cell lysis [27]. Monolaurin could inhibit germination of spores. Germination of the bacterial endospores was a degradation process that permanently stopped a dormant state. Germination was followed by growth, synthesizing novel bacterial macromolecules and converted spore sprouts into novel vegetative cells [24]. Inhibition of the spore germination by monoglycerides due to the oxygen consumption inhibition demonstrated that the cell inner membrane and its enzymes were in charge of oxygen transport [24]. Monolaurin affected respiratory activity of the cells by inhibiting enzymes involved in oxygen uptake. Oxygen affected several germination receptor-dependent pathways of germination, showing that biomolecules involved in these pathways might be sensitive to oxygen [38]. Hydrophobic surfactants were more effective than hydrophilic surfactants at killing *B. subtilis* spores [19]. Rihakova et al. reported that monoacylglycerols derived from coconut oil (mix-I and Mix-II with 97.7 and 75,1% w.w$^{-1}$, respectively) [39]. Inhibitory effects of lauroylglycerol and MIX-I on spore germination were nearly similar. In monoacylglycerols, inhibition of the spore germination increased with monoacylglycerol and 1-lauroylglycerol purity rates. Purity of the substances was associated to the rate of spore germination inhibition [39]. Surfactants with hydrophilic and hydrophobic characteristics have been shown to inhibit spore growth [19]. Counts of viable *B. subtilis* spores significantly decreased after carbonation and heat treatment during 30 days of storage in presence of specific FA esters [23]. Monolaurin seemed to include greater effects on increasing inactivation effects of carbonation and heat treatment followed by heating, compared to monocaprin [40].

### 3.3. Effects of monolaurin on hydrophobicity of the bacterial cells

Effects of monolaurin on hydrophobicity of the bacterial cells are shown in Figure 4.

Figure 4 illustrates that the presence of monolaurin in the bacterial growth media affected hydrophobicity of the bacterial cells. Presence of monolaurin significantly decreased adherence or attachment of the bacterial cells. In absence of monolaurin, adherence of *B. subtilis* bacterial cells was 56.27%. Results demonstrated higher cell hydrophobicity in *S. aureus* and *E. coli* by Gogra et al. [41]. Hydrophobicity rates of cells without DEP (diethyl phthalate) for *S. aureus* and *E. coli* were nearly 78.25 and 64.90%, showing high and moderate hydrophobicity rates [41]. Based on adherence proportion, microbial strains are grouped into highly (adherence percentage≥70%), medium (adherence percentage of 50-70%) and low hydrophobic (adherence percentage ≤ 50%) strains [41]. Therefore, this study showed that monolaurin included moderate hydrophobicity to *Bacillus subtilis*. The cell surface hydrophobicity (CSH) of microbial cells was assessed as an assessment of their attachment to n-octane using MATHS method [42,43]. This method assessed decreases in culture density following mixing and separating of hydrocarbon layers. Cells with low CSH remained on culture media, while those with higher CSH moved to the hydrocarbon layer to decrease culture density [44]. Therefore, large decreases in density of the microbial samples with high CSH occurred as well as minimal changes with low CSH [45]. Naturally, growth temperature affected the hydrophobicity of *S. aureus* cells since CSH increased with increasing the growth temperature [46]. Bacterial cells became more negatively charged as CSH increased vice versa [47].

Addition of monolaurin in nutrient broth significantly decreased hydrophobicity as shown in Figure 4.

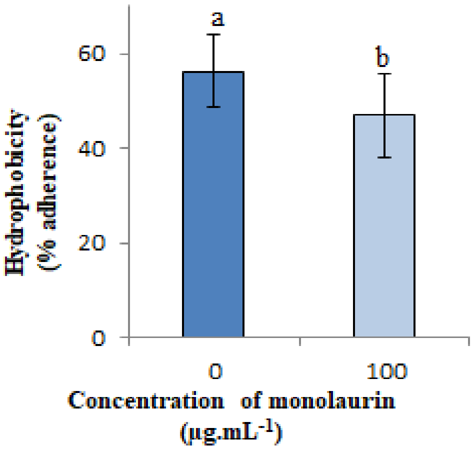

**Figure 4**. Effects of monolaurin on the hydrophobicity of *Bacillus subtilis* FNCC 0060 cells

After adding monolaurin, cell hydrophobicity of *B. subtilis* was nearly 46.94%. Amadou et al. reported that short-chain peptides (SCP) might decrease hydrophobicity of *E. coli* ATCC 8099 [48]. Furthermore, cell hydrophobicity was affected by changes in the composition of cell walls, genetic modification, temperature changes and nutrient availability [45]. Dufour et al. reported that monolaurin included a little effects on total FA changes, which increased by 21% averagely (AR01/DGVS, 27.5%; and DGRM2, 14.6%) [28]. However, it included significant effects on the FA ratio. In AR01/DGVS and DGRM2, lauric acid accounts for half of the total composition. Monolaurin changes the cellular saturated-to-unsaturated FAs ratio pf 1.4-4.35 [28]. Increasing CSH is associated to further clumping. Hydrophobic and hydrophilic characteristics of the surface of bacterial cells play significant roles in attachment to biotic and abiotic surfaces [42]. Decreases of cell hydrophobicity and increases in cell wall thickness are included in Gram-positive bacterial defense mechanisms to limit interactions with lipids [40,49]. Strains included low adhesion potentials, which could be attributed to their hydrophilic characteristics [50]. Technically, microbial hydrophobicity is important in food production and spoilage because of the interactions of the microorganisms with food components such as lipids and proteins. Bacteria with further hydrophobic surfaces are further receptive to milk fats and aroma compounds [51]. Kalamara et al. detected that *B. subtilis* NCIB 3610 formed highly hydrophobic colony biofilms [52]. Hydrophobicity is linked to localization of the BslA protein on the biofilm surface, which conferred increased resistance to biocides [52]. In general, surface positivity and hydrophobicity enhance antibacterial and antibiofilm characteristics of the magnetic nanoparticles (MNPs) [53].

### 3.4. Cell structure of *Bacillus subtilis* FNCC 0060

Figure 5 shows the TEM micrographs of *B. subtilis* FNCC 0060 cells without monolaurin and with monolaurin. Monolaurin in nutrient broth caused damages to the cell membrane of bacteria. No changes were seen in the cell cytoplasm of *B. subtilis* FNCC 0060 without monolaurin (Figure 5a). In contrast, cells cultured in presence of monolaurin showed contractions of the cytoplasm (Figure 5b).

Presence of monolaurin caused *B. subtilis* FNCC 0060 cytoplasm to shrink significantly, while junctions of the cell membrane and wall seemed increased (Figure 5B) because its small size favored insertions into the cytoplasmic membrane through electrostatic and hydrophobic interactions. Asymmetrical distribution of the lipids across the bilayer alters the intermolecular cohesion between the lipids, bending up the membrane to the point where it can be disintegrated by micellization [54]. Effects of monolaurin on cell membrane of *B. subtilis* were reported by Dufour et al. as well, who reported that the presence caused cytoplasmic membrane of *Enterococcus* (*E.*) *faecalis* DGRM2 to shrink. A cavity or space was formed, involving the cell membrane and wall, and effects of monolaurin on morphology of *E. faecalis* DGRM2 occurred due to decreases in surface permeability [28]. Zhang et al. reported that *E. coli*, *B. subtilis* and *S. aureus* cells treated with monolaurin microemulsion and incubated at 37 °C for 10 min caused cell membrane damages and disorganization of the components [20]. Wang et al. reported that surfaces of the bacterial cells became rough and cells seemed distorted after exposure to monolaurin [5]. Gram-positive bacterial cell wall structure, consisting of peptidoglycans, teichoic acids and proteins, allow hydrophobic compounds to pass through the walls into the cytoplasm [2]. Lipid (FA and monoglyceride) composites act by rupturing the bacterial cell membrane, preventing replication of DNA [55].

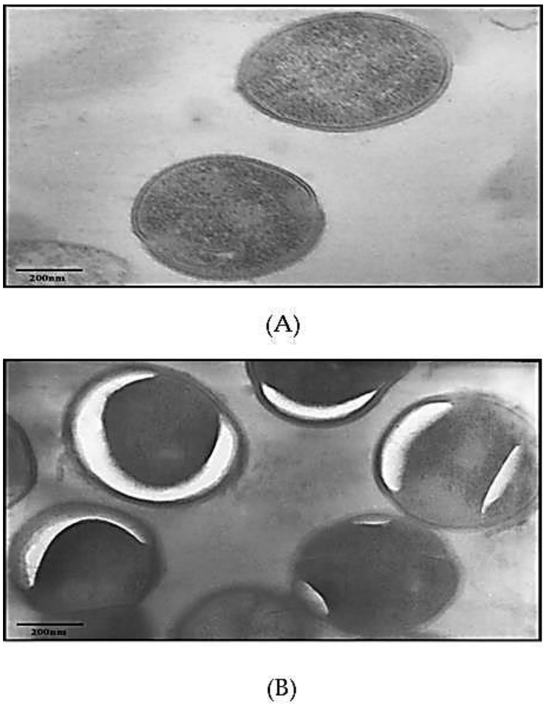

**Figure 5**. Micrographs of *Bacillus subtilis* FNCC 0060 without monolaurin (A) and with monolaurin (B)

## 4. Conclusion

In general, purified monolaurin from PKOo-PKS enzymatic glycerolysis inhibited growth of *B. subtilis* FNCC 0060. Growth of *B. subtilis* in nutrient broth with monolaurin at 100-5,000 $\mu g.ml^{-1}$ was slower than that without monolaurin. Higher concentrations in media decreased specific growth rates and increased doubling times. Spores of *B. subtilis* FNCC 0060 in nutrient broth with monolaurin of 100-1,000 $\mu g.ml^{-1}$ were germination delayed up to seven days of incubation. Presence of monolaurin significantly decreased adherence of the bacterial cells. Furthermore, cytoplasm of *B. subtilis* FNCC 0060 seemed to shrink significantly, causing cell cytoplasm damages and disorganization of the components. Therefore, monolaurin can be used as a growth inhibitor for spoilage bacteria such as *B. Subtilis* and foodborne pathogens as well as for food preservation. The antimicrobial effects can be enhanced in presence of other antibacterial compounds.

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## 6. Conflict of Interest

The authors report no conflicts of interest.

## 8. Authors Contributions

Conceptualization, T.U.; methodology, N.N.; software, N.N.; validation, T.U., C.H. and E.S.R.; formal analysis, N.N.; investigation, N.N.; resources, T.U.; data curation, T.U.; writing-original draft preparation, N.N.; writing—review and editing, T.U., C.H., E.S.R.; visualization, N.N.; supervision, T.U.; project administration, N.N.; funding acquisition, T.U. All authors have read and approved the final manuscript.

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# فعالیت ضدباکتریایی مونولائورین تخلیص شده به‌دست آمده از گلیکولیز استئارین-اولئین پالم کرنل در برابر *باسیلوس سوبتیلیس*

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## چکیده

**سابقه و هدف:** مونولائورین را می‌توان با گلیسرولیز آنزیمی مخلوط اولئین-استئارین پالم کرنل تولید کرد. مونولائورین می‌تواند به‌عنوان یک عامل ضدباکتریایی در برابر باکتری‌های گرم مثبت و گرم منفی عمل کند. مطالعات اندکی فعالیت مونولائورین را در برابر سلول‌ها و هاگ‌های *باسیلوس سوبتیلیس* بررسی کرده اند. بنابراین، هدف این مطالعه بررسی اثرات مونولارین خالص شده به‌دست آمده از گلیسرولیز آنزیمی مخلوط اولئین-استئارین پالم کرنل بر رشد، جوانه زنی هاگ، آب گریزی سطح سلول و ساختار سلولی *باسیلوس سوبتیلیس* FNCC 0060 بود.

**مواد و روش ها:** مونولائورین با استفاده از مخلوط اولئین پالم کرنل در بهترین شرایط گلیسرولیز آنزیمی تولید و با استفاده از روش حلال (هیدرالکلی) خالص سازی شد. اثرات مونولارین خالص شده بر رشد *باسیلوس سوبتیلیس* و جوانه زنی اسپور با استفاده از نوترینت براث مورد بررسی قرار گرفت. تجزیه و تحلیل آبگریزی سطح سلول با استفاده از روش چسبندگی میکروبی به هیدروکربن ها انجام شد و ساختار سلول با استفاده از میکروسکوپ الکترونی عبوری مورد مطالعه قرار گرفت. از طرح آزمایشی کاملا تصادفی استفاده و هر تیمار در سه تکرار انجام شد. برای تجزیه و تحلیل داده ها از آزمون تحلیل واریانس و چند دامنه دانکن استفاده شد.

**یافته‌ها و نتیجه‌گیری:** نتایج نشان داد که مونولائورین خالص شده رشد *باسیلوس سوبتیلیس* FNCC 0060 را مهار می‌کند. علاوه بر این، رشد *باسیلوس سوبتیلیس* در نوترینت براث با مونولائورین ppm ۱۰۰ تا ۵۰۰۰ کندتر از رشد بدون مونولائورین بود. غلظت بالاتر در محیط باعث کاهش سرعت رشد ویژه میکروبی و افزایش زمان دو برابر شدن شد. اسپور *باسیلوس سوبتیلیس* FNCC 0060 در نوترینت براث با مونولائورین ppm ۱۰۰-۱۰۰۰، جوانه زنی را تا هفت روز پس از گرمخانه‌گذاری به‌تاخیر انداخت. حضور مونولائورین به‌طور قابل توجهی باعث کاهش چسبندگی سلول‌های باکتریایی شد. علاوه بر این، به‌نظر می‌رسد که سیتوپلاسم *باسیلوس سوبتیلیس* FNCC 0060 به‌طور قابل توجهی به‌دلیل آسیب سیتوپلاسمی و به هم ریختگی اجزا، کوچک شده باشد.

**تعارض منافع:** نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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