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The potential of *Bacillus* strains isolated from the rumen content of dairy cows as natural antibacterial and antioxidant agents for broilers

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ABSTRAK

Penelitian dilaksanakan untuk mengetahui aktivitas antibakteri dan efek peningkatan antioksidan dari beberapa strain *Bacillus* yang diisolasi dari isi rumen sapi perah. Aktivitas antibakteri dari *Bacillus* terhadap bakteri *Escherichia coli* ditentukan berdasarkan metode difusi sumuran (*agar-well diffusion method*). Untuk menentukan efek peningkatan antioksidan, empat strain *Bacillus* dicampur (disiapkan sebagai *multi-strain/mixed-Bacillus*) dan digunakan untuk memfermentasi limbah obat herbal (LOH). Efeknya kemudian dibandingkan dengan efek fermentasi dari probiotik komersial *Bacillus subtilis* pada LOH dan LOH yang tidak difermentasi. Hasil penelitian menunjukkan bahwa setiap strain *Bacillus* mampu menghambat pertumbuhan *E. coli*. Fermentasi dengan *mixed-Bacillus* cenderung ($P=0,07$) meningkatkan aktivitas antioksidan dan kandungan total fenol pada LOH. Sebaliknya, fermentasi dengan *Bacillus subtilis* komersial cenderung ($P=0,07$) menurunkan aktivitas antioksidan dan total fenol LOH. Fermentasi dengan kedua starter *Bacillus* meningkatkan ($P<0,05$) kandungan protein kasar dan abu pada LOH, sedangkan lemak kasar meningkat ($P<0,05$) pada LOH yang difermentasi dengan *Bacillus subtilis*. Fermentasi baik dengan *Bacillus subtilis* ataupun *mixed-Bacillus* meningkatkan ($P<0,05$) kandungan serat kasar LOH. Sebagai kesimpulan, strain *Bacillus* yang diisolasi dari isi rumen sapi perah menunjukkan aktivitas antibakteri dan efek peningkatan antioksidan pada LOH sehingga berpotensi sebagai aditif pakan yang dapat menggantikan peran antibiotik dan antioksidan sintesis pada ayam yang dipelihara di daerah panas.

Kata kunci: antibakteri, antioksidan, Bacillus, fermentasi, limbah obat herbal

ABSTRACT

11 The study was conducted to investigate the *in vitro* antibacterial activity and antioxidant-enhancing effect of *Bacillus* strains isolated from the rumen content of dairy cows. The antibacterial activity of *Bacillus* strains was assessed against *Escherichia coli* based on the agar-well diffusion method. To assess the antioxidant-enhancing effect, the *Bacillus* strains was mixed (prepared as multi-strains) and used to ferment herbal medicine waste (HMW). The effect was compared with that of commercial probiotic *Bacillus subtilis* and non fermented HWM. Results showed that each *Bacillus* strain was able to inhibit the growth of *E. coli*. Fermentation with mixed *Bacillus* strains tended ($P=0.07$) to enhance the 2,2-diphenylpicrylhydrazyl (DPPH) radicals scavenging activity and total phenolics content of HMW. In contrast, fermentation with commercial *Bacillus subtilis* tended ($P=0.07$) to decrease DPPH radical scavenging activity and total phenol of HMW. Fermentation with both *Bacillus* preparations increased ($P<0.05$) the contents of crude protein and ash in HMW, while crude fat increased ($P<0.05$) in HMW fermented with *Bacillus subtilis*. Fermentation either with *Bacillus subtilis* or mixed *Bacillus* strains

increased ($P < 0.05$) the fiber content of HMW. In conclusion, *Bacillus* strains isolated from the rumen content of dairy cows showed antibacterial activity and antioxidant-enhancing effect, therefore, they are potential as feed additive to substitute synthetic antibiotics and antioxidants for broilers raised under tropical conditions.

Keywords: Antibacterial, antioxidant, Bacillus, fermentation, herbal medicine waste

INTRODUCTION

Broiler industry has grown substantially over the last decade in Indonesia. As a tropical country, high temperature is one of the most crucial factors in Indonesian broiler industry. Exposure to hot temperature may cause heat stress leading to performance and health problems in broiler chicks (Sugiharto *et al.*, 2017a). Heat stress may shift the normal microbial balance (increased the population of pathogenic bacteria) leading to the changes in intestinal morphology and integrity. The latter condition implies in the impairment of digestive and immunological functions of broiler intestine (Sugiharto *et al.*, 2017a; Sugiharto *et al.*, 2017b). It has commonly been practiced to use in-feed antibiotics to control intestinal pathogenic bacterial populations and improve growth performance in poultry reared under heat stress conditions (Sandikci *et al.*, 2004; Rahimi and Khaksefidi, 2006). Also, synthetic antioxidants have been used to ameliorate the detrimental effects of heat stress (oxidative stress) especially on the defence system of chicks (Sugiharto *et al.*, 2017b). It should, however, be noted that long-term administration of in-feed antibiotics and synthetic antioxidants may leave residues in broiler meats, which can risk human health. Indeed, the residue of antibiotics in broiler meat may cause antibiotic resistance (Sugiharto, 2016), while synthetic antioxidants can induce carcinogenic effect on humans (Blaszczyk *et al.*, 2013; Abreu *et al.*, 2015). Owing to this fact, any alternatives to substitute the role of in-feed antibiotics and synthetic antioxidants are essential for broiler production in the tropical countries.

Among the in-feed antibiotics substitutes, probiotic microorganisms have received the most attention from poultry nutritionists (Sugiharto, 2016). In most circumstances, probiotics are isolated from the host gastrointestinal tract. However, there has been a recent trend to use probiotic microorganisms isolated from another animal species (Puspani *et al.*, 2016) and other unconventional sources (Sornplang *et al.*, 2016). It has been known that rumen contains a huge numbers of microorganisms that provide benefits

not only for the host but also for other farm animals (Fraga *et al.*, 2014). Considering the above facts, in the earlier study, some *Bacillus* strains from the rumen content of dairy cows have been isolated and identified (unpublished data). Yet, the functional properties of these isolates remained unelucidated so far. In the present study, these *Bacillus* strains were investigated for their antibacterial activity. The latter property is crucial to control the pathogenic bacteria populations in the intestine of heat-stressed birds. Recently, there has been a considerable interest to use *Bacillus* spp., not only for probiotics, but also for enhancing the antioxidant properties of feed ingredients through fermentation. Juan *et al.* (2010) reported an enhancement of antioxidant activity, total phenolic and flavonoid content of black soybeans following fermentation with *Bacillus subtilis* BCRC 14715. Concomitantly, Sanjukta *et al.* (2015) showed enhanced antioxidant properties of soybean after fermentation with *Bacillus subtilis*. It should, however, be noted that the effect of *Bacillus*-fermentation on antioxidant activities is species/strain- and substrate-specific, as Yoon *et al.* (2015) reported a reduced antioxidant activity in black rice bran after fermentation with *Bacillus subtilis* KU3.

Herbal products have traditionally been used for human medicine for centuries. At recent times, herbal products have also been employed as antibacterial/anti coccidial agents (Muthamilselvan *et al.*, 2016) as well as for antioxidant source for poultry (Wang *et al.*, 2008). However, the use of herbal products for broiler production seems impractical as it has to compete with the human (Elfahmi *et al.*, 2014). In the recent years, herbal medicine industries have been growing considerably in Indonesia. Apart from the herbal medicine products (for human consumption), the industries produce a large amount of waste that may raise environmental problems (Soetrismanto *et al.*, 2012). Indeed, although in limited quantities, most of HMW still contains phenols, flavonoids and tannins that are antioxidant components (Kisworo *et al.*, 2016). Through *Bacillus*-fermentation, the antioxidant

properties of HMW were therefore expected to be increased and, eventually, can efficiently be used as natural antioxidants for broiler. The objective of the present study was to investigate the *in vitro* antibacterial activity and antioxidant-enhancing effect of mixed *Bacillus* strains isolated from the rumen content of dairy cows. In this study, the *Bacillus* strains were prepared in mixed-/multi-strains (instead of single-strain) when being used as a fermentation starter of HMW. This was actually inspired by data from the *in vivo* study suggesting that multi-strains *Bacillus* are more effective than single-strain in exerting beneficial effects (Chang *et al.*, 2017).

MATERIALS AND METHODS

In vitro Antibacterial Activity of *Bacillus* Strains

The antibacterial activity of *Bacillus* strains, isolated from the rumen content of dairy cows, against *Escherichia coli* ATCC 25922 was conducted according to the agar-well diffusion method (Yilmaz *et al.*, 2006) with few modifications. In brief, nutrient agar (Merck KGaA, Darmstadt, Germany) was poured into sterile Petri dish and once the agar was solidified, it was then punched with a six millimetres diameter wells. The *Bacillus* strains (i.e., *Bacillus cereus* strain SIIA_Pb_E3, *Bacillus licheniformis* strain FJAT-29133, *Bacillus megaterium* strain F4-2-27 and *Bacillus sp.*11CM31Y12) and *E. coli* were cultured separately in nutrient broth (Merck KGaA) and incubated aerobically at 38°C for 24 hours. After the centrifugation at 6,000 g for 15 min, the supernatant of each *Bacillus* strain was obtained. The suspension of *E. coli* (100 µL) was spread on the plates, and the supernatant of *Bacillus* strains (20 µL) was filled into the wells of agar plates. The plates were incubated aerobically at 38°C for 24 hours, and the diameter of the inhibition zone was determined using callipers. The assays were conducted in duplicate.

Fermentation of Herbal Medicine Waste

In this study, fermentation of HMW was conducted either with commercial probiotic *Bacillus subtilis* or mixed *Bacillus* strains as tested above. The unfermented HMW was used as a control. The probiotic *Bacillus subtilis* concentrate (contained 1×10^{22} cfu/g) was obtained from PT. Bayer Indonesia (Jakarta, Indonesia). The mixed *Bacillus* strains consisted of 6.5×10^{12} , 2.4×10^{11} , 2.6×10^{13} and 1.7×10^{13}

cfu/g for *Bacillus cereus* strain SIIA_Pb_E3, *Bacillus licheniformis* strain FJAT-29133, *Bacillus megaterium* strain F4-2-27 and *Bacillus sp.*11CM31Y12, respectively. Zeolite was used as a carrier for the bacteria.

The HMW used in the present study was obtained from PT. Sidomuncul, Tbk. (Semarang, Central Java, Indonesia). It was the by-products of “Tolak Angin” herbal medicine composing of *Foeniculli fructus*, *Isorae fructus*, *Caryophylli folium*, *Zingiberis Rhizoma*, *Menthae arvensis Herba*, *Phyllanthi Herba*, *Mel depuratum*, *Myristicae semen*, *Amomi fructus*, *Centellae Herba*, *Parkiae semen* and *Oryza sativa*. Following sterilization (using autoclave at 121°C for 15 min), 500 g of HMW was inoculated either with 1% of probiotic *Bacillus subtilis* concentrate or mixed *Bacillus* strains preparation. The mixture was then soaked with sterile water (1:1), mixed thoroughly, and incubated for four days and turned every two days. The rest of HMW was not fermented, and used as a control. The experiment was conducted in triplicate. Fermented samples were obtained for the determination of antioxidant activity and proximate compositions.

Determination of Antioxidant Activity

The determination of the 2,2-diphenylpicrylhydrazyl (DPPH) free radical scavenging activity of the samples was conducted according to Brighente *et al.* (2007) with few modifications. In brief, samples from the *Bacillus*-fermentation and control were macerated before analysis. Maceration was conducted by dissolving 0.1 g of fermented HMW in 1 mL ethanol. The mixture was sonicated and centrifuged at 6,000 rpm for 5 minutes, and the supernatant was then obtained for analysis. The supernatant (0.5 mL) was added to 2.5 mL of a solution of DPPH radicals in methanol. The mixture was shaken and let to stand for 30 minutes at room temperature. The absorbance was measured at 517 nm. The radical scavenging activity of the sample was expressed as IC₅₀ value. The following formula was used to determine the scavenging capability of DPPH radicals:

$$\text{Inhibition (\%)} = [1 - (\text{AS}/\text{ANS})] \times 100$$

Where AS was the absorbance of DPPH solution with sample and ANS was the absorbance of DPPH solution without sample. The assays were conducted in triplicates.

Determination of Total Phenolics

Total phenolics content of fermented and

unfermented HMW was determined according to Folin-Ciocalteu method (Sugiharto *et al.*, 2016) with few modifications. The supernatant as prepared above (0.5 mL), distilled water (8 mL), Folin-Ciocalteu reagent (0.5 mL, Merck KGaA) and sodium carbonate (Na_2CO_3 , 1 mL, Merck KGaA) were mixed, and incubated at room temperature for 30 minutes. Using spectrophotometer, the absorbance of the solution was measured at 765 nm. A standard curve was subsequently plotted using gallic acid. The assays were performed in triplicate.

Determination of Total Tannins

The determination of total tannins in fermented and unfermented HMW was based on colorimetric method using Folin-Denis reagent (Chanwitheesuk *et al.*, 2005). Briefly, supernatant from the fermented or unfermented HMW (0.5 mL) was added with 8 mL distilled water, 0.5 mL Folin-Denis reagent (Merck KGaA) and 1 mL sodium carbonate (Na_2CO_3). The mixture was homogenized thoroughly and incubated at room temperature for 30 minutes. The absorbance of the solution was then measured at 760 nm using tannic acid solution (Sigma-Aldrich) as a standard solution. The assays were carried out in triplicate.

Proximate Analysis

The samples of fermented HMW were analysed for proximate compositions based on the methods of the Association of Official Analytical Chemists (AOAC, 2005). The crude protein content was determined based on Kjeldahl method, while the crude fat was based on Soxhlet extraction method. The digestion method was employed to determine the crude fiber content. The content of ash in the samples was determined by ashing the samples at 550°C for 3 hours. The analyses were carried out in triplicate.

Data Analysis

The data on antibacterial activity of each *Bacillus* strain were not statistically analysed. The data of DPPH radical scavenging activity, total phenolic, tannin contents and proximate composition of fermented HMW were analysed by analysis of variance (ANOVA) using the general linear models procedure in SAS (SAS Inst. Inc., Cary, NC, USA). Significant differences ($P < 0.05$) among treatment groups were further analysed using Duncan's multiple range test.

RESULTS

Antibacterial Activity of *Bacillus* strains

The antibacterial activity of each *Bacillus* strain against *E. coli* was represented by the diameter of the inhibition zone (Table 1 and Figure 1). It was apparent that each *Bacillus* strain was able to inhibit the growth of *E. coli*.

DPPH Radical Scavenging Activity, Total phenolic and Tannin Contents in Fermented HMW

Data on DPPH radical scavenging activity, total phenolics and tannin contents in HMW are presented in Table 2. Fermentation with mixed *Bacillus* strains tended to enhance the antioxidant activity and total phenolics in HMW. Conversely, fermentation with commercial *Bacillus subtilis* tended to decrease DPPH radical scavenging activity and total phenolic in HMW. The effect of *Bacillus*-fermentation was not significant in term of tannin contents in HMW.

Proximate Compositions of Fermented HMW

The data on chemical compositions of fermented HMW are presented in Table 3. Fermentation with both *Bacillus* preparations increased ($P < 0.05$) the contents of crude protein and ash in HMW, while crude fat increased ($P < 0.05$) in HMW fermented with *Bacillus subtilis*. Fermentation either with *Bacillus subtilis* or mixed *Bacillus* strains increased ($P < 0.05$) the fiber content of HMW.

DISCUSSION

In response to the ban of in-feed antibiotics in broiler production, nutritionists are now searching for safer alternative feed additives for broiler chickens. Although probiotics has been considered the safest and most promising alternative, the efficacy of such additive on broiler health and performance is variable depending on the species and strains of the probiotic microorganisms (Sugiharto, 2016). In this reason, the search for more effective and consistent probiotic microorganism is still necessary to support the sustainable broiler production especially in the tropical countries. In the earlier study, some strains of *Bacillus* from the rumen content of dairy cows have been isolated and identified. To follow up, this present study investigated an *in vitro* antibacterial activity, which is one of the most important probiotic

Table 1. Diameters of Inhibition Zone Exhibited by *Bacillus* Strains Against *E. coli*^a

<i>Bacillus</i> strains	Diameters of inhibition zone (mm)
<i>Bacillus cereus</i> strain SIIA_Pb_E3	11.3 ± 0.45
<i>Bacillus licheniformis</i> strain FJAT-29133	12.7 ± 0.67
<i>Bacillus megaterium</i> strain F4-2-27	13.3 ± 0.35
<i>Bacillus</i> sp. 11CM31Y12	12.5 ± 0.47

^aValues are mean ± SD

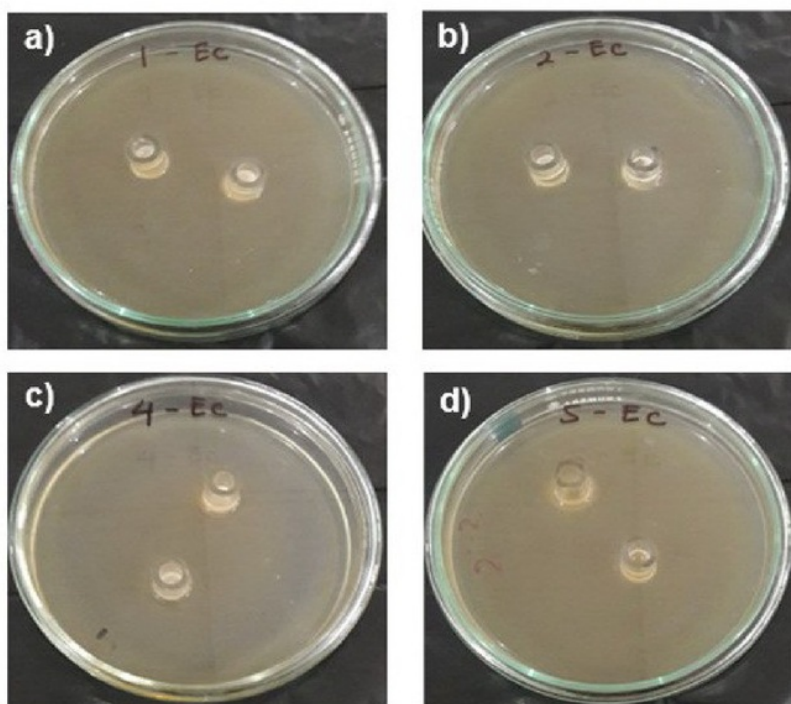


Figure 1. The inhibition zone exhibited by *Bacillus* strains against *E. coli*; a) *Bacillus cereus* strain SIIA_Pb_E3, b) *Bacillus licheniformis* strain FJAT-29133, c) *Bacillus megaterium* strain F4-2-27, and d) *Bacillus* sp. 11CM31Y12

properties, of these *Bacillus* strains. It was apparent in our result that four isolates of *Bacillus* strains were able to inhibit the growth of *E. coli*. Concomitant with our results, Yilmaz *et al.* (2006) in the previous study showed the antibacterial activity of some *Bacillus* spp. strains isolated from the soil against both Gram-positive and

Gram-negative bacteria. The antibacterial activity of *Bacillus* species was also reported by Amin *et al.* (2015) against *Staphylococcus aureus* (PTCC 1112), *Shigella dysenteriae* (PTCC 1188), *E. coli* (PTCC 1396), *salmonella typhi* (PTCC 1609), and *Corynebacterium diphtheriae* (ATCC 27010). The mechanism through which our isolates inhibited

Table 2. DPPH Radical Scavenging Activity, Total Phenolic and Tannin Contents in HMW Fermented by Different Starters^a

Starters	IC ₅₀ ^b (µg/mL)	Total phenolics (mg/100 g)	Total tannins (mg/100 g)
Control	262 ± 64.4	12.2 ± 4.50	5.60 ± 0.89
<i>Bacillus subtilis</i>	314 ± 18.0	6.40 ± 0.30	5.23 ± 0.21
Mixed <i>Bacillus</i> strains	197 ± 2.81	14.5 ± 0.70	6.37 ± 0.94

^aValues are mean ± SD

^bIC₅₀ is identified as the effective concentration at which the 2,2-diphenylpicrylhydrazyl (DPPH) radicals were scavenged by about 50%. A higher of DPPH radical scavenging activity is associated with a lower IC₅₀ value.

Table 3. Chemical Composition of HMW Fermented by Different Starters (as Dry Matter Basis)^a

Starters	Crude protein (%)	Crude fat (%)	Crude fiber (%)	Ash (%)
Control	10.7 ± 0.10 ^b	2.81 ± 0.13 ^b	22.8 ± 0.49 ^c	4.97 ± 0.19 ^c
<i>Bacillus subtilis</i>	11.5 ± 0.47 ^a	3.83 ± 0.55 ^a	28.2 ± 0.51 ^a	5.96 ± 0.29 ^a
Mixed <i>Bacillus</i> strains	11.3 ± 0.27 ^a	3.15 ± 0.24 ^b	26.2 ± 0.89 ^b	5.47 ± 0.17 ^b

^aValues are mean ± SD

the growth of *E. coli* was not clear, but Amin *et al.* (2015) proposed that bacitracin produced by *Bacillus* spp. may be responsible as antibacterial substances. Taking this finding into the consideration, the *Bacillus* strains in this study may therefore be employed as natural antibacterial agents that can substitute the role of in-feed antibiotics in broiler rations.

As mentioned earlier, administration of synthetic antioxidants to broiler chickens has been attributed to the potential carcinogenic effect on humans as consumers (Blaszczyk *et al.*, 2013; Abreu *et al.*, 2015). With food safety reason, broiler farmers are now encouraged to use natural antioxidants to protect the birds from the detrimental effects of oxidative stress (Sugiharto *et al.*, 2017a), one of which is herbal products (Wang *et al.*, 2008). However, the use of such products for broilers may compete with the human need for medicine (Elfahmi *et al.*, 2014). Considering the continuous production of HMW in Indonesia and the antioxidant properties in

HMW, the present study aimed to increase the antioxidant properties of HWM through *Bacillus*-fermentation. Indeed, our present data showed that fermentation with mixed *Bacillus* strains tended to increase the antioxidant activity of HMW. This finding was in accordance with Juan *et al.* (2010) showing an enhancement of antioxidant activity, total phenolic and flavonoid content in black soybeans following fermentation with *Bacillus subtilis* BCRC 14715. Conversely, fermentation with commercial *Bacillus subtilis* tended to lower antioxidant activity of HMW in the current study. Corresponding to this, Yoon *et al.* (2015) reported that fermentation with *Bacillus subtilis* KU3 reduced antioxidant activity in black rice bran. In these regards, this study confirmed that the effect of *Bacillus*-fermentation on the antioxidant properties was species- and strain-specific (and perhaps substrate-specific). In the case of the enhanced antioxidant activity after fermentation (with mixed *Bacillus* strains), such bacterial fermentation may increase the

production and release of phenolic compounds with high antioxidant activity (Yao *et al.*, 2010). This inference was supported by the fact that fermentation with mixed *Bacillus* strains increased the content of total phenolics in HMW. In nature, phenolic compounds are bound with sugar, and during fermentation these complex components were degraded into free phenols with high antioxidant properties (Adetuyi and Ibrahim, 2014). In addition to phenolic compounds, the enhanced antioxidant activity in mixed *Bacillus* strains-fermented HMW may be attributed to the increased total tannins in HMW (though the values were not statistically significant). Note that tannin may contribute to the antioxidant capacity of materials (Sugiharto *et al.*, 2016). Overall, fermentation with mixed *Bacillus* strains could be the method to optimize the use of HMW as natural antioxidant sources for broiler chickens raised under hot ambient temperature.

As mentioned earlier, fermentation with commercial *Bacillus subtilis* tended to decrease DPPH radical scavenging activity and total phenols in HMW. The definite explanation for the decreased antioxidant activity in commercial *Bacillus subtilis*-fermented HMW was not known, but the degradation of phenolic compounds during fermentation with *Bacillus subtilis* may be one of the reasons (Paliyath *et al.*, 2011). Note that phenolics are reactive compounds that may be destructed by enzymatic and/or non-enzymatic reactions during fermentation process (Hunaefi and Smetanska, 2013). With regard to *Bacillus subtilis*, Hasan and Jabeen (2015) reported that inoculation with *Bacillus subtilis* isolated from soil was able to degrade phenol in culture medium. Concomitantly, Nair and Prakash (2017) showed the capability of *Bacillus subtilis* SP3 in degrading phenolic compounds *in vitro*.

Recently, there has been a considerable interest to produce functional feeds by fermenting the feed or feed ingredients using selected probiotic microorganisms (Sugiharto *et al.*, 2015; 2017c). In such case, fermented products may not only rich in probiotics and their metabolite products, but also having improved nutritional values. In the present study, fermentation with both mixed *Bacillus* strains and commercial *Bacillus subtilis* increased the contents of crude protein and ash in HMW, while crude fat increased only in HMW fermented with *Bacillus subtilis*. This finding was in accordance with that of reported by Wongputtisin *et al.* (2014) showing the increased crude protein and ash contents in

soya bean hull after fermentation with *Bacillus subtilis*. Concomitantly, *Bacillus amyloliquefaciens*-fermentation increased the contents of crude protein in rice bran (Supriyati *et al.*, 2015) and *Bacillus sphaericus*-fermentation increased the fat content of soybean (Jeff-Agboola and Oguntuase, 2006). *Bacillus*-fermentation may, therefore, result in product (fermented HMW) that can supply the nutrients to broiler chickens, in addition to the supply of functional components such as antibacterials and antioxidants as discussed earlier. In contrast to Supriyati *et al.* (2015), *Bacillus*-fermentation increased the fiber content of HMW in the present study. However, our finding was concomitant with Jeff-Agboola and Oguntuase (2006) and Wongputtisin *et al.* (2014) showing an increased fiber content of soybean and soya bean hull after fermentation with *Bacillus sphaericus* and *Bacillus subtilis*, respectively. The definite reason for the increased fiber content in fermented HWM was not clear so far. In the study of Jeff-Agboola and Oguntuase (2006), the increased fiber was accompanied by the decreased carbohydrate content in soybean. This may suggest that fermentation degraded the complex carbohydrate (polysaccharides) into simpler fiber (oligosaccharides), resulting in the increase and decrease in crude fiber and carbohydrate contents, respectively.

CONCLUSION

It could be concluded that *Bacillus* strains isolated from the rumen content of dairy cows had antibacterial property and antioxidant-enhancing effect. It was, therefore, possible to use these *Bacillus* strains as feed additive to substitute the role of synthetic antibiotics and antioxidants for broiler chickens, especially those are raised under tropical conditions.

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