THE EFFECT OF BACILLUS subtilis ON THE BACTERIAL CONTENT IN RABBITS CAECI
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ABSTRACT
In the present study, the effect of B. subtilis (10⁷ cfu/gm) supplemented to the basal diet of two groups of New Zealand rabbit of 28 days (n=15/group) on the count of commensal microflora (Bifidobacteria and Lactobacilli spp.) and some opportunistic pathogens (E. coli and C. perfringens type A) in the rabbit caeci was investigated. Results indicated that the count of Bifidobacteria and Lactobacilli spp. are significantly increased at all intervals (7, 14, 28 and 40 days post supplementation) in the supplemented group compared with non-supplemented group with P<0.001. On the other hand, there is a significant decrease in the count of E. coli and C. perfringens specially at 40 days post supplementation from (5.8 log 10 cfu ±0.06) to (3.1 log 10 cfu ±0.07) and from (4.8 log 10 cfu ±0.09) to (2.3 log 10 cfu ±0.09) respectively with P<0.001.

B. subtilis proved to be tolerant to the simulated gastric and intestinal juice for one hour (100% tolerance). However, after two hours, the tolerance to the gastric juice decreased to 70%, with no effect of the artificial intestinal juice on the viability of the organism. In vitro, the antimicrobial effect of B. subtilis on C. perfringens type A by well diffusion method showed an inhibition zone of 10 mm. Results of in vitro effect of B. subtilis on C. perfringens count showed that at 10⁷ dilutions of C. perfringens, its count was reduced 2.1x10⁵ to 3x10⁴ after adding B. subtilis, while at dilution of 10⁸, the count was reduced from 2.4x10⁵ to 7x10⁴cfu/ml after adding B. subtilis. It could be concluded that B. subtilis can be used as a probiotic in rabbits ration due to its ability to increase the commensal microflora count beside its antibacterial effect against some opportunistic pathogens.

Keywords: Rabbit, Probiotics, B. subtilis, C. perfringens, E. coli, Caeci.

INTRODUCTION
In developing countries, livestock production provides food and play an important role in economic improvement and is considered as a significant income source for many family farms. In the past, the use of antibiotic growth promoters (AGPs) in large scale to animal feed has contributed to the increase in livestock production. However, the over-use of AGPs resulted in the emergence of multidrug-resistant microorganisms and consequently prohibited by the European Union which introduced a total ban on AGPs in 2006 (Castanon, 2007).

The prohibition on the sub-therapeutic use of antibiotics in animal feed resulted in decreased animal production (Cheng et al., 2014) due to higher rates of infections in livestock and has also increased the risk of food-borne diseases in consumers (Hao et al., 2014). Several replacements/alternatives have been proposed to overcome the problems associated with the ban of AGPs on livestock production (Cheng et al., 2014). The use of probiotics is considered as one strategy that has proven effective.

In the last decade, several strains of some Bacillus spp. are currently used as probiotic dietary supplements in animal feeds. Bacillus is a genus of Gram-positive, aerobic or facultatively anaerobic, endospore-forming bacteria. Bacillus spp. are saprophytes commonly associated with soil, water, dust and air. In addition to an intestinal origin, Bacillus probiotics can also be isolated from other sources, including food, plants, marine algae, aquatic habitats and soil (Mingmongkolchaisri and Panbangred, 2018).

Spore-forming-Bacillus spp. Among a variety of bacterial species used as probiotics has been identified as an appropriate probiotic because its spores are resistant to harsh conditions and long-term storage...
at ambient temperature as well as an inhibition for pathogenic bacterial growth (Chen et al., 2013). Compared with other probiotic microorganisms, Bacillus spp. have many advantages as probiotic supplements in animal feed because of their ability to produce spores. In addition to heat stability, Bacillus sp. spores have the ability to survive the low pH of the gastric barrier. They can reach the small intestine to exert their probiotic properties (Cutting, 2011).

It was found that Bacillus spores germination occur in the small intestine (Cartman et al., 2008), and may induce their protective effect in the host through metabolically active mechanisms, such as secretion of antimicrobial substances and/or competition with pathogenic bacteria for essential nutrients (Duc et al., 2004). In addition to stimulating positive changes in the intestinal microflora, diarrhea recovery, increased body weight gain and feed efficiency were observed in the host when the animals were fed B. Subtilis (Jiraphocakul et al., 1990).

Probiotics have been used widely in poultry production. Several studies were conducted to investigate the beneficiary effect of B. subtilis probiotics against S. Enteritidis or Clostridium perfringens (La Ragione and Woodward 2003), C. perfringens and Enterobacteriaceae (Jeong and Kim 2014), Campylobacter (Guyard-Nicodeme et al., 2016) and Salmonella colonization in broilers (Knap et al., 2011). An intensive system of rabbit production, especially during the weaning period, can cause many physiological and environmental stresses. These problems result in concentration and spreading of pathogens such as E. coli, coccidians and epizootic rabbit enteropathy which have adverse effects on growth performance, feed efficiency and rabbit health status (Bovera et al., 2010 and Licois et al., 2000).

Therefore, our study was conducted to investigate the beneficiary effect of B. subtilis as a feed supplement in rabbit feeding, and its effect on some commensal and opportunistic pathogenic microorganisms such as E. Coli and C. perfringens in rabbit caeci (in vivo) and to study its effect on C. perfringens type A (in vitro).

**MATERIALS AND METHODS**

**Bacterial strains and Media**

1. *Bacillus subtilis* strain

A reference commercial strain of *B. subtilis* (ATCC 10876) was kindly obtained from Food and Meat Hygiene Department in Animal Health Research Institute. It was aerobically cultured in trypton soya broth (TSB) supplemented with 1% (w/v) yeast extract (TSBYE) and incubated for 18 hours at 37°C aerobically.

2. *Clostridium perfringens* type A strain

It was isolated from a case of enteritis in rabbits, the pathogenicity and toxicity of this isolate were tested for lethality and dermonecrotic reaction in Swiss mice and guinea pigs respectively (willis, 1977). *C. perfringens* isolate was anaerobically cultured in thioglycolate broth and incubated for 18 hours at 37°C. Both *B. subtilis* and *C. perfringens* broth cultures were centrifuged (3000xg for 10 min) and washed using sterile phosphate buffer saline (PBS) pH 7.4. In cell culture medium a final concentration of 107 cfu/ml was re-suspended for further testing.

**Experimental design**

**Study the effect of B. subtilis on bacterial content count in rabbit caeci:**

To study the effect of *B. subtilis* on bacterial content in rabbit caeci in vivo, 30 healthy weaned New Zealand White male rabbits (28 day-old) were divided into 2 groups consisting of 15 rabbits each. Group 1 were fed with a basal diet supplemented with 107 cfu/gm basal diet of *B. subtilis*, while rabbits of group 2 were fed with a basal diet only and served as a control group. All groups ran contemporaneously in a battery with sufficient room temperature, feed, and ventilation until the end of the experiment (6 weeks).

Intestinal samples were collected at 7, 14, 28 and 40 days post supplementation (3 samples/time) from each group for enumeration of some commensal and opportunistic pathogenic microorganisms as described by (Guo et al., 2017) as follows: The intestinal contents were serially diluted in 0.85% sterile saline solution and each dilution was plated in duplicate onto selective agar for each microorganism (eosin methylene blue for enumeration of *E. coli*, trypsin sulphate cycloserine agar for *C. perfringens*, MRS agar for *Lactobacillus spp.*, and Bifidobacterium agar medium for *Bifidobacterium spp.*).

**Survival of Bacillus subtilis in a simulated gastrointestinal juice**

A simulated gastrointestinal juice was made to investigate the survival of *B. subtilis* in these juices. The suspension of 2.3×107 cfu/ml of *B. subtilis* were inoculated into Trypton Soya Broth (TSB) and pH was adjusted to 2.5 by adding a hydrochloric acid solution with 1% pepsin (as gastric juice) or to 7.2 with 1% trypsin (as intestinal juice) and incubated at 37°C for up to two hours. At 0, 1 and 2 hours of incubation, 100 µl of the culture were removed and spread onto trypton soya agar plates in duplicate for cell number estimation as described by (Guo et al., 2017).

**Antimicrobial activity of B. subtilis**

The well diffusion method was performed on agar using cultured broth. The target strain *C. perfringens* in a concentration of (107 cfu/ml) was incorporated into agar (1% w/v) plates, mixed for uniformity and poured onto plates to solidify. Overnight culture of *B. subtilis* (107 cfu/ml) was transferred to holes (5mm in diameter) punched into
the agar plates. The plates were then incubated anaerobically at 37°C for 24 hours and the antimicrobial inhibition zone was recorded as described by (Zhang et al., 2012).

**Effect of B. subtilis on C. perfringens count (in vitro)**

Clostridium perfringens suspension in PBS was prepared as $10^5$ cfu/ml using McFarland tube from which tenfold dilution was made, and 1 ml of each dilution was dispensed in sterile Petri dishes then 10 ml of molten tryptose sulphate cycloserine agar (TSC) was poured, mixed well and left to solidify before another layer was poured. All *C. perfringens* suspension dilutions were seeded with 0.1 ml of overnight culture of *B. subtilis* ($10^7$ cfu/ml) and incubated for 1 hour at 37°C then cultured onto TSC agar as mentioned before. After solidification, the plates were incubated anaerobically at 37°C for 24 hours then enumerated as described by (Guo et al., 2017).

**Statistical Analysis**

Experimental data were assessed by one-way analysis of variance (ANOVA)-Duncan test using SPSS software statistical program (windows version 20.0, USA) to observe mean differences (SPSS 20.0, 2014). Data were expressed as mean ± SE when (n=5) and were regarded as significant when (P≤0.05).

**RESULTS**

**Detection of B. subtilis effect on bacterial content in rabbit caeci**

It was found that the beneficial microflora e.g. *Bifidobacterium* and *Lactobacillus* spp. obviously increased in group treated with *B. subtilis* and the opportunistic pathogenic organisms count were decreased in this group. On the other hand, the control group nearly showed stability in the count of all microorganisms as shown in Table (1).

**Survival of Bacillus subtilis in simulated gastric and intestinal juices**

*Bacillus subtilis* ATCC 10876 exhibited a good probiotic property in vitro including artificial gastric juice and intestinal juice. The strain can tolerate the alkalinity of the intestinal juice till 2 hours; however, it may show less tolerance with the acidity of gastric juice after 2 hours as shown in Table (2).

**Effect of B. subtilis on C. perfringens type A count (in vitro)**

It was observed that, at $10^3$ dilutions, the count of *C. perfringens* culture was $2.1 \times 10^5$ cfu/ml, however, when *B. subtilis* was added, the count of *C. perfringens* decreased to $3 \times 10^4$ cfu/ml (Figs. 1 and 2). On the other hand; when *C. perfringens* culture was at $10^2$ dilutions, its number reached $2.4 \times 10^5$ cfu/ml and decreased to $7 \times 10^3$ cfu/ml after treated with *B. subtilis* (Figs.3 & 4).

**Antimicrobial activity of B. subtilis**

The sensitivity of *C. perfringens* against *B. subtilis* on tryptose sulphate cycloserine agar (TSC) was made and a zone of inhibition of about 10 mm has been formed. A sterile Trypricase soya yeast extract (TSY) broth was used as control negative in another well (Fig.5).

Fig. 1: count of *C. perfringens* diluted $10^3$ times without adding *B. subtilis* culture ($2.1 \times 10^5$ cfu/ml).

Fig.2: count of *C. perfringens* diluted $10^3$ times after adding *B. subtilis* culture ($3 \times 10^4$ cfu/ml)

Fig.3: The count of *C. perfringens* diluted $10^2$ times without adding *B. subtilis* culture ($2.4 \times 10^5$ cfu/ml).
Fig. 4: The count of *C. perfringens* diluted $10^2$ times after adding *B. subtilis* culture ($7 \times 10^3$ cfu/ml).

Fig. 5: The sensitivity of *C. perfringens* against *B. subtilis* on TSC agar. (a) showing a zone of inhibition of 10 mm. (b) Control negative using sterile TSB broth.

Table 1: Enumeration of commensal and pathogenic bacteria in caeci of rabbits.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Count of commensal and pathogenic opportunistic microorganisms in (log$_{10}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microorganisms</td>
<td>Group 1</td>
</tr>
<tr>
<td></td>
<td><em>(B. subtilis-supplemented)</em></td>
</tr>
<tr>
<td>day post supplementation</td>
<td>7</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>5.4±</td>
</tr>
<tr>
<td><em>C. perfringens</em></td>
<td>4.5±</td>
</tr>
<tr>
<td><em>Bifidobacterium</em></td>
<td>7.8±</td>
</tr>
<tr>
<td><em>Lactobacillus</em></td>
<td>9.0±</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01, ***p<0.001

Table 2: Survival rate of *B. subtilis* at artificial gastric juice and intestinal juice (%) (The mean of count).

<table>
<thead>
<tr>
<th>Juice</th>
<th>Time</th>
<th>0 hour</th>
<th>1 hour</th>
<th>2 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric juice</td>
<td>2.3×10$^7$</td>
<td>100% (1.8×10$^7$)*</td>
<td>70% (1.6×10$^6$)**</td>
<td></td>
</tr>
<tr>
<td>Intestinal juice</td>
<td>2.3×10$^7$</td>
<td>100% (1.9×10$^7$)*</td>
<td>100% (1.7×10$^7$)*</td>
<td></td>
</tr>
</tbody>
</table>

* count not changed (complete tolerance)  
** count changed (partial tolerance)
DISCUSSION

Probiotics are safe alternative to antibiotics which will improve growth performance and act as therapeutically or prophylactically drugs, as antibiotics disturbs the normal microbial balance and beneficial bacteria (Simoneit et al., 2014) and (Sullivan et al., 2001). B. subtilis are considered to be suitable probiotics as they produce essential nutrients such as amino acids and vitamin K and B12, promoting growth performance and proliferation of beneficial bacteria as well (Cutting, 2011 and Zhou et al., 2013). The effectiveness of probiotics depends largely on the dose ingested and bacterial strains, therefore, it is essential to determine the minimal effective dosage of probiotic strains (Mingmongkolchai and Panbangred, 2018).

In this study, the role of B. subtilis as a dietary probiotic in rabbit ration was investigated to determine its effect on some opportunistic pathogens as well as some beneficial commensal bacteria in the gut of rabbits. It was found that B. subtilis had a significant inhibitory effect on the count of pathogenic bacteria in the gut of rabbits as the count of E. coli as well as C. perfringens were decreased significantly throughout the experiment. These results are on line with Phuoc and Jamikorn (2017) who investigated the role of B. subtilis supplement on feed efficiency, growth performance and microbial population in the distal gastrointestinal tract of weaning rabbits and found that the average intestinal coliform populations were lowest (P<0.05) in the rabbits supplemented with B. subtilis than control.

Additionally; suppression of colonization and persistence of S Enteritidis or C. perfringens was demonstrated in pre-dosing newly hatched specific pathogen-free chicks with a suspension of 1×10⁸ spores of B. subtilis PY79⁹. Moreover, S. Enteritidis shedding was also reduced significantly in the pre-dosed birds for the 36-days duration of the experiment (La Ragione and Woodward 2003). Similarly, Jeong and Kim (2014) showed that the number of C. perfringens and Enterobacteriaceae in the excreta is reduced when birds were fed B. subtilis C-3102.

Reduction in shedding of Campylobacter is observed in broilers fed B. subtilis C-3102, (Guyard-Nicodeme et al., 2016) and a significant reduction in Salmonella colonization in broilers fed with either B. cereus var toyoii (Toyocerin) (Vila et al., 2009) or B. subtilis DSM 17299(Knap et al., 2011). Supplementation of B. subtilis PB6 to broilers also resulted in a reduction in intestinal C. perfringens counts (Jayaraman et al., 2013).

It was found that the count of beneficial commensal bacteria (Bifidobacterium spp. and Lactobacillus spp.) significantly increased in B. subtilis supplemented group (P<0.001). Guo et al. (2017) found that the survival rate of the rabbits fed with B. subtilis was significantly higher than those in the control groups post infected with E. coli. It is worth to say that B. subtilis strains that do not have any probiotic activity or killed cells cannot produce digestive enzymes, vitamins and antibacterial substances, which were produced by probiotic. For that, it is speculated that strains without probiotic activity have no significant effects on growth performance, intestinal flora and disease resistance. The ability of B. subtilis to inhibit the adhesion of enterotoxic E. coli to the surface of intestinal epithelial cells and S. Enteritidis through competitive exclusion (CE) mechanism was reported by other investigators (Thirabunyanon and Thongwittaya, 2012; Ye et al., 2013).

Probiotics should also be excellent in adapting to acidic conditions in the stomach and alkalinity in the intestine to influence the gastrointestinal tract. The ability of the spores to survive at low pH of the gastric barrier and reach the small intestine to produce the probiotic effects was documented by Barbosa et al. (2005) and Cutting (2011) that accord with our findings as it was found that B.subtilis can tolerate the alkalinity of the simulated intestinal juice in a rate of 100% after 1 and 2 hours incubation. Meanwhile, the organism can tolerate the acidity of the artificial gastric juice in a rate of 100% after 1 hour and the rate decreased after 2 hours to 70%. Germination of Bacillus spores in the small intestine has been reported in several studies (Cartman et al., 2008). Their beneficiary effect in the animal host may be attributed to the active metabolic mechanism, such as competition with pathogenic bacteria for essential nutrients and/or secretion of antimicrobial substances (Duc et al., 2003). It is worthy to mention that before using a probiotic strain to demonstrate its beneficial effect on animal host; the in vivo and in vitro studies must be investigated (Mingmongkolchai and Panbangred, 2018).

In the present study; in vitro study at dilution 10⁴, the count of C. perfringens type A reached 2.1×10⁵ cfu/ml, however, when B. subtilis was added, the count reduced to 3.0×10⁴ cfu/ml. On the other hand, when C. perfringens culture was at 10² dilutions, the count reached 2.4×10⁵ cfu/ml and reduced to 7.0×10⁴ cfu/ml after treated with B. subtilis. Several antimicrobial compounds such as bacteriocins, lipopeptides, bacteriocins like inhibitory substances and surfactin are known to be produced by genus Bacillus members. These compounds are typically active against Gram-positive bacterial pathogens, but some display activity against Gram-negative bacterial pathogens as well as fungal pathogens (Kerr, 1999; Teo and Tan, 2005; Khochamit et al., 2015). Moreover, by conducting
antimicrobial activity of *B. subtilis* against *C. perfringens*, a 10 mm inhibition zone was produced indicating the antimicrobial efficacy of *B. subtilis*. These results are in agreement with Teo and Tan, (2005) who examined the antimicrobial activity of *B. subtilis* against *C. perfringens* ATCC 13124.

**CONCLUSIONS**

*Bacillus subtilis* is safe and reliable animal probiotic with great potential to be used as an alternative to antimicrobial drugs which is significant in the context of antibiotic abuse in food and animal production. Dietary supplementation of *B. subtilis* in rabbit ration will decrease the bacterial load of pathogens and subsequently improves the growth rate of animals.

**Declaration of Competing interest**

On behalf of all authors, I hereby declare that no conflict of interest may interfere with the publication of the manuscript.

**REFERENCES**


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