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In vivo and in vitro study on the effect of Bacillus subtilis and its byproducts on Clostridium perfringens Type A

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The objectives of this study were to screen the possible effects of *B. subtilis* and its soluble byproducts against *C. perfringens*, a causative agent of necrotic enteritis (NE) in chickens. The use of *B. subtilis* strain was found to be inactive in vitro against *C. perfringens* but its cell filtrate byproducts produced after growth of *B. subtilis* at 37°C with medium pH adjusted at 5.0, having inhibitory effect in the form of inhibitory zones; measured inhibition of 12 mm. The produced anticlostridial factor was not affected by heat treatment at 70, 100 or 121°C for 15 minutes. The study recorded the responses of broiler chickens to oral administration of *C. perfringens* and the possible antagonistic effects of *B. subtilis* and its cell-free filtrate byproducts in vivo. Results revealed that *B. subtilis* decreased the severity of intestinal necrotic lesions produced after oral inoculation of *C. perfringens* and the suggested anticlostridial effect was more clear when the cell-free filtrate produced by growing *B. subtilis* at 37°C with pH 5.0 was added to feed at a dose of 20 ml/kg of ration.

Substantial progress has been made in the development of probiotics, prebiotics and synbiotics, which are effective in increasing and maintaining the population of lactic acid bacteria in the intestine (Klein *et al*., 1998).

Bacillus subtilis is considered generally recognized as safe and has found application in the feed industry (Salminen *et al*., 1996). Necrotic enteritis, an enterotoxemic disease caused by *Clostridium perfringens*, leads to the development of necrotic lesions in the gut wall, resulting in mortality of poultry (Paulus and Rockepusch, 1996).

This disease is also multifactorial with complex and partially unknown epidemiology and pathogenesis (Kaldhusdal, 2000). Studies have shown increase in the concentration of *C. perfringens* cells in the gastrointestinal tract has been correlated with necrotic enteritis (Craven *et al*., 1999).

The objectives of this study were to screen the effect of *Bacillus subtilis* as a probiotic against *C. perfringens* Type A, the causative agent of necrotic enteritis in poultry as well as the possible effect of *B. subtilis* byproduct as anticlostridial factor.

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Materials and Methods

Bacterial strains and culture conditions

Bacillus subtilis strain. This strain is standardized strain deposit No. DSM17299. It was inoculated into tryptic soya broth (Oxoid) supplemented with yeast extract (6 g/liter) (TSBYE broth) for 24 hours at 37°C then streaked onto tryptic soya agar supplemented with yeast extract and incubated for 24 hours at 37°C to obtain pure fresh colonies (Teo and Tan, 2006).

Clostridium perfringens Type A strain. This strain was previously isolated from typical cases of necrotic enteritis in broilers and completely identified biochemically according to Koneman *et al*., (1992). This strain was also characterized by molecular techniques according to Yoo *et al*., (1997).

In vitro studies

Antagonistic assays. The strain of *Bacillus subtilis* was grown in TSBYE broth and incubated at 37°C. Isolated colony of *C. perfringens* grown onto sheep blood agar was inoculated into 10 ml of thioglycolate broth (FTB) (Oxoid) at 37°C under anaerobic conditions using anaerogen pak (Oxoid). An overnight culture of *C. perfringens* was streaked (perpendicularly) onto the surface of TSAYE agar using a sterile cotton swap according to Teo and Tan (2006). The overnight culture of *B. subtilis* was streaked across the same agar plate

bisecting the streak line of *C. perfringens* and the plate was incubated at 37°C under 5% CO_2 for 24 hours. Antagonistic effect of the test organism on *C. perfringens* was determined by the appearance of clear zone surrounding the junctions of the streak lines (Teo and Tan, 2006).

Well diffusion assays. It was used according to Teo and Tan (2006). Molten TSAYE medium containing 0.7% agar at 45°C was inoculated with an 18h culture of *C. perfringens* to obtain a final concentration of approximately 10^4 to 10^5 organisms per ml. Ten milliliters of the seeded agar was then dispensed aseptically into sterile petri dishes containing 20 ml of solidified TSAYE medium. Upon solidification of both agar layers, the surface of the agar was perforated using a sterilized cork borer to create wells of 8 mm in diameter. The wells were filled with 200 µl per well of the test samples and the plates were incubated at 37°C for 18h under anaerobic conditions. The diameters of the zones of inhibition were measured. Each well diffusion assay used for testing an antimicrobial factor with various parameters was conducted 3 times in duplicate.

Effect of growth temperature and pH on the production of anticlostridial factor by B. subtilis. *B. subtilis* was grown aerobically in TSBYE broth at 37, 45 and 50°C for 18h with automatic shaking every 2 hours then the cultures were filtered through 0.45 sterile filters to obtain cell-free filtrate. On the other hand, cells of *B. subtilis* were grown in TSBYE broth adjusted to pH 4.0, 5.0, 6.0 and 7.0 and incubated aerobically at 37°C for 18h with shaking every 2 hours. All cells of *B. subtilis* were removed by filteration through 0.45 sterile filters. The negative controls were sterile TSBYE broth adjusted to pH 4.0, 5.0, 6.0, or 7.0. All inoculated plates were incubated anaerobically at 37°C for 18h.

Sensitivity of B. subtilis byproducts to temperature. Fifty milliliter of each of the cell-free filtrates of *B. subtilis* grown at 37°C and pH 4.0, 5.0, 6.0 and 7.0 were heated at 70, 100 and121°C for 15 minutes. Each heat treated filtrate was dispensed into wells containing TSAYE medium and screened for anticlostridial activity. The inhibitory zones around the wells were determined after anaerobic incubation at 37°C for 18h.

***In vivo* study**

Experimental infection with C. perfringens. Commercial broilers (Ross) were used. A total of 80 birds were divided into four equal groups:

The first group. Birds in this group were orally inoculated daily for 7 days, with 1 ml (2.0×10^8CFU/ml) of an overnight culture of *C. perfringens* between 14 and 21 days of age (Olkowski *et al*., 2006).

The second group. Birds in this group were fed for 2 weeks (between 14 and 28 days of age) commercial ration contained cells of *B. subtilis* added at an inclusion rate of 10^9CFU/kg of feed (Teo and Tan, 2006).

The third group. Birds in this group were fed (between 14 and 28 days of age) commercial ration contained *B. subtilis* cell-free filtrate obtained after growth of *B. subtilis* in TSBYE broth at 37°C at pH 5.0. To each kilogram of ration, 20 ml of the cell-free filtrate were added and mixed well immediately before use.

The second and third groups of chickens were additionally orally inoculated daily, with 1 ml of 2.0×10^8CFU/ml of an overnight culture of *C. perfringens* between 14 and 21 days of age.

The fourth group. acts as control non infected group.

No antibiotic growth promoter was supplemented in the diet of the negative control group except for maduramicin-ammonium (Alpharma). Enough lighting was provided during the night to assure continuous feeding and water intake. On day 29, all birds were euthanized and subjected to gross postmortem evaluation according to (Calnek *et al*., 1997).

Results

Anticlostridial effect of *B. subtilis in vitro*. After 24 h of incubation, the antagonistic effect of *B. subtilis* on the growth of *C. perfringens* could not be observed.

Effect of growth temperature and pH on the production of anticlostridial factor. By growing of *B. subtilis* at various growth temperatures, no zone of inhibition was observed with cell-free filtrates extracted from cultures grown at 45 and 50°C. On the other hand, there was no zone of inhibition with *B. subtilis* filtrate grown in TSBYE broth at pH 4.0. However with growth at pH 5.0, 6.0 and 7.0, various degrees of anticlostridial activities were observed (Table 1). The maximum anticlostridial activity was observed at pH 5.0. No zone of inhibition was detected with negative controls using sterile TSBYE broth at pH 4.0, 5.0, 6.0 and 7.0.

Effect of temperature on anticlostridial activity. Very small or no significant decreases in anticlostridial activities of the filtrates were observed when they were heated at 70, 100 and

121°C for 15 minutes (Table 1).

Experimental infection

First group. Chicken of this group showed no mortalities. Gross lesions were apparent in the duodenum, jejunum, ileum and ceca in 60.0% of chickens. The intestinal wall appeared thin and friable. Intestinal mucosa was lined by yellow or greenish pseudomemberanes I 14 out of the 20 inoculated chickens. In areas with more advanced necrotic lesions, the intestinal mucosa was covered with a brownish, diphtheritic pseudomemberanes. The main clinical signs were decreased appetite, reluctance to move, diarrhea and ruffled feathers.

Second group. Intestinal necrosis was less advanced, the mesenteric vessels were engorged with blood, and the mucosa was lined by yellow membranes. No mortalities were recorded.

Third group. There were no clear changes could be seen in intestinal mucosa observed except mild, mostly focal gross changes in duodenum in the form of gray thickened mucosa.

Forth group. Mild, mostly focal changes in duodenum of control birds were recorded.

Table (1): The anticlostridial activities of *B. subtilis* byproducts under different cultural and incubation factors.

Treatment		Mean zones of inhibition
Growth temperature	37°C	8 mm
	45°C	0.0 mm
	50°C	0.0 mm
pH of growth medium	4.0	0 mm
	5.0	12 mm
	6.0	10 mm
	7.0	8 mm
Heat	70°C	8 mm
	100°C	7 mm
	121°C	7 mm

Discussion

This study was based on previous studies which demonstrated that probiotic microorganisms adhere to and colonize the epithelial cells of the gastrointestinal tract and their beneficial effects include competitive exclusion of pathogenic strains of *E. coli* (Watkins *et al*., 1982), *Campylobacter jejuni* (Morishita *et al*., 1997) and *Salmonella Entertidis* (Pascual *et al*., 1999); enhancing the growth and viability of beneficial gut microflora (Hosoi *et al*., 2000) and improved digestion and absorption of nutrients (Thomke and Elwinger, 1998) in chickens. Other criteria used include production of antimicrobial factors (Salminen *et al*., 1996). A number of lactic acid bacteria have been shown to exhibit various degrees of antimicrobial activities against clostridium species (West and Warner, 1988; Alander *et al*., 1999).

In the present study, strain of *B. subtilis* (deposit No. DSM17299) was tested for antagonism toward local isolate of *C. perfringens* Type A isolated from typical cases of necrotic enteritis in broiler chickens.

The in vitro antagonistic effect of *B. subtilis* on the growth of *C. perfringens* could not be observed meanwhile findings obtained showed that filtrates of the bacillus strain contained antimicrobial factor against *C. perfringens* when it grown at pH 5.0, 6.0 and 7.0 at incubation temperature of 37°C and the maximum anticlostridial activity was observed at pH 5.0. Production of bacteriocins by Bacillus species has been reported by (Von Tersch and Carlton, 1983; Jansen and Hirsehmann,1944; Pattnaik *et al*., 2001). Results of Teo and Tan (2006) revealed the anticlostridial bacteriocin of *B. subtilis*.

The inhibitory activity of the anticlostridial factor was maintained after heating at 70, 100 or 121°C for 15 minutes (Table 1). Similar findings were described by (Baquero and Moreno, 1984; Teo and Tan, 2006). The maximum antimicr-obial activity was observed when cells of *B. subtilis* were grown in broth at pH 5.0 and incubated at 37°C to disagree with the results of Teo and Tan (2006) which indicated that the maximum activities were observed at pH 6.2.3

After experimental infection, the *in vivo* study clarified that no mortalities were recorded. The first group which inoculated orally with *C. perfringens* showed postmortem lesions in the form of thin friable intestinal wall, the mucosa was lined by greenish pseudomembranes or brownish diphtheritic pseudomembranes meanwhile, group which received *C. perfringens* orally and *B. subtilis* in feed recorded less advanced intestinal necrosis with yellow membranes covering the intestinal mucosa. The birds which received cell-free filtrate ob *B. subtilis* grown at 37°C with pH 5.0, recorded no obvious intestinal changes except mild focal changes in duodenum. The obtained results indicate the role of *B. subtilis* byproducts in exhibiting antimicrobial activities against *C. perfringens* as reported by (Salmenin *et al*.,

1996). Because of the undetected clinical signs, the lesions observed in this study can be classified as sub-clinical. The lesions observed in this study are remarkably similar to those described by (Kaldhusdal and Hofshagen,1992; Olkowski *et al*., 2006).

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الآثار المثبطة لعصيات الباسيلس ستلس ومنتجاتها على المطثيات الحاطمه النوع أ

تهدف هذه الدراسه لتوضيح أثار عصيات الباسيلس ستلس ومنتجاتها على ميكروب المطثيات الحاطمه المسبب للالتهاب المعوى التنكرزى فى الدواجن وقد اتضح ان استخدام عصيات الباسيلس ستلس النشطه كان غير مثبطا لنمو ميكروبات المطثيات الحاطمه عند استخدام المنتجات التى أفرزتها (والتى انتجت بعد نموها عند درجة حرارة 37م ورقم هيدروجينى 5) كان لها تأثير مثبط لنمو المطثيات الحاطمه محدثه درجة تثبيط قدرها 12مليمتر وقد ثبت أن منتجات الباسيلس ستلس لم تتأثر عند تعرضها لدرجات حرارة 100،70، 120لمدة 15 دقيقة وقد شملت هذه الدراسة أيضا استبيان ا لتأثيرالمثبط لميكروب الباسيلس ستلس ومنتجاته عند تجريعه لبدارى التسمين والتى قد سبق عدوتها صناعيا بميكروب المطثيات الحاطمه وقد اتضح أن عصيات الباسيلس ستلس كان لها تأثير ايجابى في خفض درجة التنكرز المعوى وقد تم الحصول على نفس الأثر الايجابى لمنتجات الباسيلس ستلس عند انمائها فى درجة حرارة 37م ورقم هيدروجيني 5.