Original Article

Characterization and Probiotic Properties of *Bacillus* Strains Isolated from Broiler

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Abstract

In order to isolate local strain of spore-forming bacteria having probiotic properties, 164 isolates of the spore-forming bacteria were isolated from 152 samples including duodenum, jejunum, ileum and cecum collected from 38 local backyard chickens raised in the northern part of Thailand. These isolates were preliminarily screened for antibiotic susceptibility and antimicrobial acitivity. Only antibiotic-susceptible isolates with antimicrobial activity were further characterized for the ability to resist certain harsh environments. Identification of the potential isolates was carried out by morphological and biochemical studies, followed by analysis of 16S rDNA sequence. Of these isolates, only one strain designated T3-1 showed antimicrobial activity against *Clostridium perfringens* ATCC 15191 and was susceptible to antibiotics widely used in medical treatment. Based on its morphology, biochemical property and 16S rDNA sequence, T3-1 was identified as *Bacillus* sp. Spore of this strain was resistant to simulated gastric and small intestinal juices, high temperature up to 100^{o}C and could survive in water containing 5 ppm chlorine residues for 120 min. In addition, Bacillus sp. T3-1 was demonstrated to have the ability to colonize Caco2 cells as well as to produce cellulase. These findings demonstrated that *Bacillus sp.* T3-1, a local spore forming isolate, has probiotic properties which can be further developed as poultry feed additive.

Keywords: *Bacillus* sp., poultry feed additive, probiotic, spore-formers

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บทคัดย่อ

การศึกษาลักษณะสมบัติและคุณสมบัติโพรไบโอติกของสายพันธุ์บาซิลลัสที่แยกจากไก่เนื้อ

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การวิจัยครั้งนี้มีวัตถุประสงค์เพื่อคัดแยกแบคทีเรียสายพันธุ์ท้องถิ่นที่สามารถสร้างสปอร์และมีคุณสมบัติเป็นโพรไบโอติก การทดลองเริ่มโดยสุ่มเก็บตัวอย่างไก่พื้นบ้านจำนวน 38 ตัว ตัดแยกชิ้นตัวอย่างจากลำไส้เล็กส่วนต้น ส่วนกลางและส่วนปลาย รวมทั้งไส้ตันของไก่แต่ละตัว รวมทั้งสิ้น 152 ตัวอย่าง นำตัวอย่างทั้งหมดมาแยกแบคทีเรียสร้างสปอร์ จากนั้นทำการศึกษาความไวของสายพันธุ์ต่อยาปฏิชีวนะและความสามารถในการต้านแบคทีเรียก่อโรคของสายพันธุ์ที่แยกได้ คัดเลือกเฉพาะสายพันธุ์ที่ไม่ดื้อต่อยาปฏิชีวนะและมีฤทธิ์ต้านเชื้อทดสอบไว้ศึกษาคุณสมบัติการเป็นโพรไบโอติก การพิสูจน์เอกลักษณ์ของแบคทีเรียสร้างสปอร์อาศัยการศึกษาทางสัณฐานวิทยา คุณสมบัติทางชีวเคมีและลำดับเบสของยีน 16S rDNA ผลการศึกษาพบว่า จากแบคทีเรียที่สร้างสปอร์ที่แยกได้ทั้งหมด 164 สายพันธุ์ มีเพียงสายพันธุ์รหัส T3-1 ที่ไวต่อยาปฏิชีวนะที่ใช้กันแพร่หลายในทางการแพทย์ และสามารถต้านทาน *Clostridium perfringens* ATCC 15191 ได้ การจัดจำแนกเชื้อระบุว่า T3-1 เป็นเชื้อ *Bacillus* sp. สปอร์ของเชื้อดังกล่าวทนต่อน้ำย่อยเทียมของกระเพาะ และลำไส้เล็ก ทนความร้อนชื้นได้ถึง 100 องศาเซลเซียส และอยู่รอดในน้ำที่มีปริมาณคลอรีนเข้มข้น 5 พีพีเอ็ม ได้นาน 120 นาทีนอกจากนี้ *Bacillus* sp. T3-1 ยังสามารถยึดเกาะผิวเซลล์ Caco-2 รวมทั้งสร้างเอนไซม์เซลลูเลสได้ด้วย ผลการวิจัยครั้งนี้ แสดงให้เห็นว่า *Bacillus* sp. ซึ่งเป็นสายพันธุ์แบคทีเรียสร้างสปอร์สายพันธุ์ท้องถิ่นมีคุณสมบัติเป็นโพรไบโอติกที่สามารถนำไปพัฒนาเป็นสารเสริมอาหารสำหรับการเลี้ยงไก่

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Introduction

Removal of the antibiotics from feed can make poultry more susceptible to infection by certain pathogens (e.g. *Escherichia coli, Clostridium perfringens* and *Salmonella sp.*) resulting in poor performance and serious damage to poultry industry. As a consequence, researches on safe and effective alternatives to antibiotics for disease prevention and growth enhancement have been extensively carried out (AL-Sultan, 2003; Wekhe et al., 2007; Ghazala and Ali, 2008). Regarding these works, probiotics, live microbial feed beneficial to animal health, have been considered as an alternative to be used as growth promoting and prophylactic products. Several studies indicated that broilers fed with probiotics showed greater resistance to pathogen infection as well as better performance than the control group (Jin et.al., 1998; Teo and Tan, 2006).

Most of the probiotics commercially available consist of a variety of microorganisms, particularly *Lactobacillus sp., Bifidobacterium sp., Streptococcus sp., Enterococcus sp.* (Patterson and Burkholder, 2003). However, the major weak point of these species which makes them not applicable for being used as probiotics in poultry farming and animal feed industry is their sensitivity to harsh environments. Then, spore-formers become the probiotic of interest due to their extreme resistance to heat and chemicals (Nicholson et. al., 2002; Setlow, 2006; Cartman et. al., 2007). This intrinsic feature offers many advantageous impact to animal feed industry including resistance to production process, ease in distribution and storage and longer shelf–life over a wide range of temperatures.

Barbosa et al. (2005) revealed that many species of *Bacillus* including *B. subtilis, B. pumilis, B. licheniformis, B. megaterium* etc. could be isolated from fecal materials collected from broilers raised in different farms. All spores were tolerant to simulated gastrointestinal tract conditions. There was the evidence showing that probiotic strain *B. subtilis* 3

possessed anti-*Helicobacter pylori* (Pinchuk et. al., 2001).

Nevertheless, the application of spore based probiotic products is still not widely accepted in poultry farming in Thailand, particulary due to inconsistent efficiency of the product usage on farms. It should be noted that those spore probiotics available for farmed poultry were from either laboratory strains or exogenous strains of *Bacillus* sp. Some studies revealed the different biological properties between wild type and laboratory strains of *B. subtilis* (Branda et. al., 2001) or among the same species from different origins (Barbosa et. al, 2005). Hence, the difference in origin of the strains can be the reason for the failure of spore based probiotic products usage on farms. In this study, we focused on isolating and identifying of local spore-forming bacteria from broilers raised in Thailand. Characterization of biological properties desirable for probiotic agents were also performed.

Materials and Methods

Indicator strains and growth condition: *Salmonella* Enteritidis DMST 15676, *S.* Typhimurium DMST 17242, *S.* Gallinarum DMST 15968, *Escherichia coli* O157, *Clostridium perfringens* DMST 15191, and *Campylobacter jejuni* DMST 15190 were obtained from the National Institute of Health, Department of Medical Sciences, Ministry of Public Health. Stock cultures of the indicator strains were stored in culture medium with 40% glycerol at -80°C. When needed, *Salmonella* strains and *E. coli* were grown in Tryptic Soy Broth (TSB) pH 7.4 at 37°C, 250 rpm whereas *Cl. perfringens* DMST 15191 and *C. jejuni* DMST 15190 were anaerobically cultured in Wilkins Charlgren medium (WC) pH 7.4 at 37°C for 48 hrs.

Source of samples for isolation: Thirty-eight healthy broilers with approximately 2 months of age were randomly taken from 19 backyard farms located in the northern part of Thailand. Two chickens were selected as a representaive sample from each farm. Sections of duodenum, jejunum, ileum and cecum were aseptically and separately excised from each chicken at postmortem.

Isolation of spore–forming bacteria and growth conditions: Spore-forming bacteria were isolated from each sample by method previously described (Barbosa et al., 2005) with slight modifications. Briefly, each sample was initially mixed with buffered peptone-water in 1:1 ratio before thoroughly mixing by the stomacher (AES Laboratoire Model Mix 2, France). The sample was 10–folded diluted in TSB pH 7.4 and WC broth pH 7.4. Incubation was conducted at 37°C for 24 hrs with the exception that all sample containing WC broths were incubated in anaerobic jar (BBL Gaspak, Cockeysville, MD. USA). After incubation, the cultures were heated shock at 80°C for 30 min in order to select against vegetative cells. Subsequent isolation was carried out by streaking heat treated cultures on isolation media including TSA for aerobic spore-formers and WC agar for anaerobic spore-formers. Colonies with different morphological features were randomly picked, purified and kept at -80°C in either TSB or WC broth containing 40% glycerol.

Otherwise indicated, suspension of vegetative cells or spores used throughout this study was prepared as following. The suspension of vegetative cells was prepared by growing spore-forming isolates in isolation liquid media at 37°C with vigorous agitation at 250 rpm for 18-24 hrs. The culture was centrifuged at 7000xg, 4°C for 10 min. Cell pellets were washed once with phosphate buffer saline (PBS), subsequently suspended in equal volume of PBS and used freshly.

Spore suspension was prepared by heating 3-day old culture grown in Schaeffer's sporulation medium (DSM) broth at 80°C for 30 min in order to eradicate vegetative cells. The heated culture was immediately put on ice for 20 min. Spores were collected by centrifugation at 7000xg for 10 min at 4°C, washed twice with PBS and then suspended in PBS to the final concentration of 10^8 to 10^9 spores/ml. The spore suspension was kept at 4°C for further experiments.

Antibiotic susceptibility test: The susceptibility of spore-forming isolates to antibiotics used for human medication was determined by disk diffusion assay according to the Clinical and Laboratory Standards Institute (CLSI) guideline (Clinical and Laboratory Standards Institute, 2003). Types of antibiotic disk (Oxoid, England) tested were amoxicillin (10 µg), penicillin G (30 µg), rifampicin (30 µg), tetracycline (30 µg) and vancomycin (30 µg). The disk diffusion breakpoints indicating susceptibility to amoxicillin, penicillin G, rifampicin, tetracycline and vancomycin are ≥ 18 mm, ≥ 15 mm, ≥ 20 mm, ≥ 19 mm and ≥ 17 mm, respectively. The antibiotic sensitive isolates were selected for screening of antimicrobial activity.

Screening for antimicrobial activity: Colony overlay assay (Pugsley, 1985) was used to determine antimicrobial activity of spore-forming isolates against the indicator strains. Five microliters of an overnight culture of each spore-forming isolates were spotted on isolation media (3 spots/plate) and incubated at 37°C for 24 hrs. Bacterial cells in each spot were killed by exposure to chloroform vapor for 30 min prior to 20 min of aeration to remove chloroform residue. Inoculum of the indicator strains was prepared by calibrating the overnight culture to a turbidity of 0.5 McFarland standard. Then, 0.1 ml of the inoculum was thoroughly mixed with 5 ml isolation medium containing 0.7% agar and the mixture was subsequently overlaid on the chloroform treated spots. Any spore-forming isolate showing inhibition zone around the spot was considered to have antimicrobial activity and subsequently used for hemolysis assay.

Hemolysis of red blood cells: Each spore-forming isolates was streaked on Blood agar base (Difco) supplemented with 5% sheep blood and grown at 37°C for 24 hrs. The presence of clear zone around colonies indicated lysis ability of those colonies and was considered as the positive result.

Determination of probiotic characteristics: Antibiotic sensitive, non hemolytic spore-forming isolates with antimicrobial activity were further determined for essential probiotic properties.

Survival to simulated gastric juice and simulated intestinal juice: Vegetative cells and spores of each isolates were comparatively studied for their resistance to simulated digestive juices as previously described (Barbosa et al., 2005). Briefly, suspension of spores or vegetative cells were inoculated into simulated gastric juice pH 2 containing isotonic buffer (1.24% K_2HPO_4, 0.76% KH_2PO_4, 0.1% Trisodium citrate, 0.1% $[NH_4]_2SO_4$) and 0.4% pepsin (Sigma) or simulated intestinal juice (isotonic buffer pH 7 with 0.3% bile salt [Sigma]). Incubation was carried out at 37°C with vigorous shaking at 250 rpm. Viable count was done at 0, 30, 60, 90 and 120 min after exposure by drop plate technique on the appropriate isolation media. Resistance of each isolates was reported as % survival.

Resistance to heat: Ability of the isolates to resist wet heat was carried out at 80°C and 100°C, a range of temperatures used for pelleting process in animal feed industry. The suspension of vegetative cells or spores were heated up to the desirable temperature and held for 180 min. Survival cells or spores were determined at various time intervals; 0, 15, 30, 60 and 180 min by viable count on the suitable isolation medium.

Resistance to Chlorine solution: The spore suspension was inoculated into sterile distilled water containing 3 and 5 ppm chlorine residue and left at room temperature. Samples were taken after 0, 5, 15, 30, 60 and 120 min, serially diluted in normal saline containing 0.1% sodium thiosulphate to neutralize chlorine toxicity and counted on the suitable isolation medium.

In vitro adherence to epithelial cells: Human intestinal epithelial cell line used was Caco-2 (ATCC HTB–37) purchased from the American Type Culture Collection. All media as well as supplements were from Gibco, Invitrogen Lifescience, USA, otherwise noted. The isolates were in vitro test for the ability in adhering to Caco-2 cells based on the methods previously described (Moroni, 2006). In brief, semiconfluent monolayers of Caco-2 cells were prepared by seeding Caco-2 cells grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM *L*–glutamine, 1% non essential amino acids and 1% antibiotic solution (100 U/ml penicillin and 100 µg/ml streptomycin) in 6-well plates (Nunc, USA) and incubated at 37°C under 5% (v/v) CO_2 atmosphere. For the assay, the monolayers were washed twice with PBS and replenished with antibiotic free DMEM for 1 hr. After removal of medium, each well was infected with approximately 10^6 colony forming unit (cfu) of vegetative cells or spores suspended in antibiotic free DMEM for 1h. Then, the monolayers were washed twice with PBS to eliminate free cells or free spores. The adherent cells or spores were recovered from each well by scraping and counted on the isolation medium. Adhesion activity was expressed as the number of adherent cells divided by total number of culture infected, multiplied by 100.

Screening for cellulase activity: Cellulase producing isolates were screened using plate screening method as described by Hankin and Anagnostakis (1977). Briefly, 10 µl of supernatant of the overnight culture grown in Nutrient Broth were spotted on minimal medium containing 0.5% carboxymethyl cellulose (SIGMA). After 24-hr incubation at room temperature, the plates were flushed with 0.1% (w/v) Congo red solution and kept for 1 min before washing the plates with 0.1M NaCl. Clear zone around the spot indicates cellulase activity.

Identification of the isolates: The spore-forming isolates were initially identified by macroscopic and microscopic appearances, followed by biochemical test using the API 50 CHB strips (bioMérieux, France) and APILAB Plus software for result analysis. Oxidase activity was tested with 3% hydrogen peroxide solution (Sigma) whereas catalase activity was determined using Bactident Oxidase strips (Merck, England).

Analysis of 16S ribosomal DNA (rDNA) sequence: Genomic DNA was extracted according to Sambrook et al. (1999) and used as a template for amplifying 16S rDNA. PCR amplification was carried out with Takara Ex Tag® (Takara BIO. Co., Japan) using forward universal primer (5'-GAGTTTGATCATGGCTCAG-3') and reverse universal primer (5'-CGCTTACCTTGTTAGCGACTT-3'). PCR product was purified by QIAquick purification kit (Qiagen Gmbh, Germany) and subjected to the sequencing reaction using ABI Prism^{Rm} Bigdye^{Tm} Terminator V. 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA).

Statistical analysis: All data collected were subjected to analysis of variance (ANOVA) using SPSS program version 11.5. Significant differences between treatment means were separated using the Duncan's multiple range test with a 5% probability.

Results

Isolation of spore–forming bacteria from chicken gastrointestinal tract: With the hope to obtain an effective and safe spore-forming probiotics suitable for poultry farms in Thailand, source of samples and origin of strain became the important criteria of the sampling plan. Statistics of livestock between 2007 till 2009 reported by Information Technology Center, the Department of Livestock Development (www. dld.go.th) indicated that the northern part of Thailand was one of the areas where poultry farms either contract or backyard farms are mainly located. As a consequence, sampling sites were focused on those areas. Although backyard reared chickens could be a source of various pathogens because of the rearing style, they were still chosen due to several reasons. Firstly, they were raised without antibiotic supplemented feed or antibiotic treatment resulting in decreased chance to get antibiotic resistant strains

upon isolation. Secondly, their feed consisted of a variety of cereal grains, plant seeds or herbs. Finally, they were always raised in close contact to the ground. Therefore, the intestinal microflora of these backyard chickens were supposed to be more diverse compared to those raised in commercial farms.

By heat shock method, 164 colonies representing different morphologies on various isolation media and growth conditions as well as sampling sites were selected and purified. It was found that 78 colonies were aerobic spore formers grown on TSA whereas 86 colonies were detected on WC agar incubated anaerobically. When grown in DSM agar for 48-72 hrs, all colonies formed spores which could be confirmed by malachite green staining and visualization under light microscope. It should be noted here that morphology of colonies obtained from duodenal, jejunal, ileal or cecal samples from the same bird were similar (data not shown).

Antibiotic susceptibility test: It should be noted here that zone diameter interpretive standard specific for spore-forming bacteria has not yet been available in any standard guidelines. Hence, the zone diameter breakpoints determined by the general recommendation of the Société Française de Microbiologie French Society for Microbiology (http://www.sfm.asso.fr/Wuk.html) and zone diameter interpretive chart of BBL™ Sensi-Disk™ Antimicrobial Susceptibility Test Disks based on CLSI (2003) were used to interpret the results of susceptibility test in this study. The zone diameter breakpoints of amoxicillin, penicillin G, rifampicin, tetracycline, and vancomycin for spore-forming bacteria are ≥ 18 mm, ≥ 15 mm, ≥ 20 mm, ≥ 19 mm and ≥ 17 mm, respectively.

In this study, the susceptibility of 164 spore–forming isolates to 5 commonly used antibiotics for human medication was determined by disk diffusion assay. Only 62 isolates (36 isolates of aerobic spore formers and 26 isolates of anaerobic spore formers) were susceptible to amoxicillin, penicillin G, rifampicin, tetracycline and vancomycin. The remaining isolates showed diverse susceptibility profiles. Some isolates were susceptible to either amoxicillin or penicillin G or both whereas the others were susceptible to 3 to 4 antibiotics. Two isolates of anaerobic spore formers were resistant to all antibiotics tested. Comparison of percentage of the isolates susceptible to each antibiotic and ranges of inhibition zones detected between aerobic spore-formers and anaerobic spore–formers indicated that the aerobic spore-formers seemed to be slightly more resistant than the latter ones (Table 1). It should be noted that aerobic spore-formers seemed to be more resistant to vancomycin whereas anaerobic spore–formers showed less susceptible to tetracycline.

Table 1. Antibiotic sensitivity profiles of 164 spore-forming isolates determined by disk diffusion assay.

Types of antibiotic disk	Percentage of isolates susceptible to each antibiotic	
	Aerobic spore formers (ranges of inhibition zone)	Anaerobic spore formers (ranges of inhibition zone)
Amoxicillin (10 µg/disk)	85.9% (18–38 mm)	96.2% (18–50 mm)
Penicillin G (30 µg/disk)	73.1% (15–37 mm)	93.6% (15–49 mm)
Rifampicin (30 µg/disk)	61.5% (20–32 mm)	79.5% (20–42 mm)
Tetracycline (30 µg/disk)	70.5% (19–44 mm)	54.5% (19–50 mm)
Vancomycin (30 µg/disk)	64.1% (17–40 mm)	88.5% (17–44 mm)

Screening for antimicrobial and hemolytic activities

Except *S.* Gallinarum causing fowl typhoid, the other indicator strains are not only the causative agents of poultry diseases but they are also zoonotic agents (Meslin, 1997). Sixty two antibiotic sensitive isolates were tested for their antagonistic effects to these indicator strains by colony overlay assay. None of these isolates possessed antimicrobial activity to *S.* Enteritidis DMST 15676, *S.* Typhimurium DMST 17242, *S.* Gallinarum DMST 15968 and *E. coli* O157. In contrast, there were 9 isolates exhibiting antimicrobial activity against either *Cl. perfringens* DMST 15191 or *C. jejunii* DMST 15190 or both indicator strains (Table 2). Out of 9 isolates, 3 isolates namely T1-2, T2-3 and T3-1 showed antimicrobial effect to *Cl. perfringens* DMST 15191 whereas isolates T8-2 and M 4-1 showed inhibitory activity to *C. jejuni* DMST 15190. The other 4 isolates able to generate inhibition zones against both *Cl. perfringens* DMST 15191 and *C. jejuni* DMST 15190 were T7-5, T11-4, W1-1 and W1-2. Our results were well correlated with the previous work which exhibited that strains of *Bacillus* isolated from broiler gastrointestinal tract showed no inhibitory activity to *Salmonella* spp. and *E. coli* (Barbosa et. al., 2005).

Lysis of red blood cells is one of the mechanisms causing pathogenesis in bacterial infection. Hence, these 9 isolates were subsequently assayed for hemolytic activity using 5% sheep blood agar in order to check the possibility of these isolates to be pathogenic. It was found that isolate T2-3 and T3-1 did not lyse the red blood cells. As a consequence, isolates T1-2, T7-5, T8-2, T11-4, M4-1, W1-1 and W1-2 were excluded from the study.

Table 2. Antimicrobial activity of antibiotic sensitive spore formers against certain pathogens assayed by colony overlay assay.

Isolates[a]	Antimicrobial activity against different indicator strains[b]					
	Cl. perfringens DMST 15191	*S.* Enteritidis DMST 15676	*S.* Typhimurium DMST 17242	*S.* Gallinarum DMST 15968	*E. coli* O157	*C. jejuni* DMST 15190
T 1-2	+	–	–	–	–	–
T 2-3	+	–	–	–	–	–
T 3-1	+	–	–	–	–	–
T 7-5	+	–	–	–	–	+
T 8-2	–	–	–	–	–	+
T 11-4	+	–	–	–	–	+
M 4-1	–	–	–	–	–	+
W 1-1	+	–	–	–	–	+
W 1-2	+	–	–	–	–	+

[a]Isolates T1-2, T2-3, T3-1, T7-5, T8-2 and T11-4 were aerobic spore formers whereas M 4-1, W1-1 and W1-2 were anaerobic spore formes.

[b] +: inhibition zone around chloroform treated spot was observed.

- : no inhibition zone around chloroform treated spot was detected.

Identification of isolate T2-3 and T3-1: Colony morphology of T2-3 and T3-1 grown on TSA for 24 hr were similar; creamy, large, opaque and rough texture with irregular edge. Microscopic examination of isolate T2-3 and T3-1 revealed Gram-positive, rod shaped cells ranged from 2-4 µm in length and 0.5 -1 µm in width. Oval endospores were found centrally in the cells. Both T2-3 and T3-1 could produce catalase but not oxidase.

Biochemical properties of both isolates were studied using the API 50 CHB kit. It was revealed that isolate T2-3 and T3-1 showed nearly identical biochemical profiles. The results of identification by API 50 CHB kit were confirmed by analysis of 16S rDNA sequence. The full lenght of 16S rDNA (around 1,500 bp) amplified from genomic DNA of isolates T2-3 and T3-1 were aligned to those deposited in GenBank using Basic local Alignment Search Tool (BLAST) Program. The 16S rDNA sequences of T2-3 and T3-1 presented 97% and 98% identity to *Bacillus* sp, respectively. Based on the identification results, T3-1 was selected for the following experiments.

Determination of probiotic characteristics: Once ingested, spores of *B. subtilis* could germinate in the gastrointestinal tract indicating that spores functioned in the host body through their vegetative cells (Cartman et. al., 2008). As a consequence, both vegetative cells and spores of *Bacillus* sp. T3-1 were determined for their ability to resist harsh stresses in the gastrointestinal tract. According to the digestive system of chicken, feed will be mixed with gastric juice containing acid and enzymes when they are in proventiculus and gizzard before entering to small intestine where bile salt and pancreatin are present. Duration of transit time of feed in the proventiculus and gizzard takes around 90 min where that in the small intestine is about 120 min. Therefore, survival of *Bacillus* sp. T3-1 in the digestive juice was assayed within 120 min of contact time which were more realistic.

Bacillus sp. T3-1 spores were highly tolerant to both simulated gastric juice and simulated intestinal juice. Survival rate of spores at each time interval was equivalent to 100%. In the case of vegetative cells, resistance of *Bacillus* sp. T3-1 to the simulated gastric juice was similar to its spores because the cell numbers of *Bacillus* sp. T3-1 enumerated throughout the course of assay were not significantly different from those of spores. In contrast, vegetative cells of *Bacillus* sp. T3-1 were more sensitive to the simulated intestinal juice than spores, especially at 120 min of contact time in which % survival of the vegetative cells was significantly reduced to 86.4% (Figure 1).

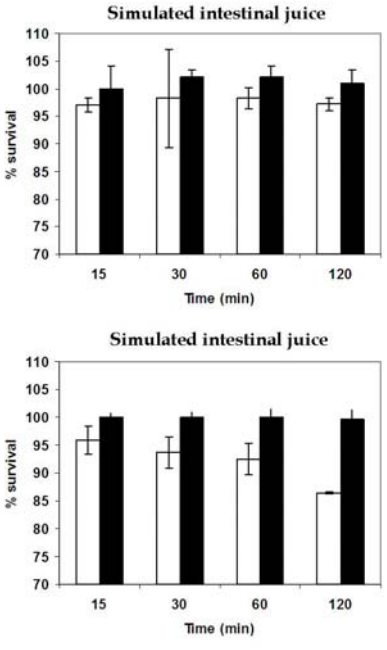

Figure 1. Survival of *Bacillus* sp. T3–1 vegetative cells (□) and spores (■) in digestive juices. The data were the means of triplicate experiments and the error bars indicated standard error.

Resistance to heat is the other desirable characteristic of probiotic intended to use in feed industry. In our study, it was found that wet heat at 80°C did not affect spore viability at any contact time tested whereas the number of vegetative cells were

reduced approximately 2.6 log after the first 15 min of contact time and remained stable till the end of the assay (Figure 2A). Effect of the wet heat on viability of vegetative cells and spores could be clearly seen at 100°C. The vegetative cells of *Bacillus* sp. T3-1 were dramatically reduced after 15 min of contact time and were completely destroyed at 60 min of exposure time onwards. Although spores seemed higher tolerant to wet heat at 100°C than vegetative cells, their viability did gradually reduce when the contact time increased. After 180 min of contact time, log number of spores remained around 4.55±0.17 cfu/ml (Figure 2B).

According to the biosecurity program applied in poultry farming, the water used in poultry houses including drinking water must be treated with chlorine. In general, the chlorine residue remained in the drinking water is about 3 to 5 ppm. Furthermore, *Bacillus* sp. T3-1 will be mixed with poultry feed in the form of spores. Therefore, only spores of *Bacillus* sp. T3-1 were tested for their resistance to chlorine residues at the level of 3 and 5 ppm. It was found that spores of *Bacillus* sp. T3-1 were resistant to chlorine residues up to 5 ppm even after 120 min of exposure time (Table 3).

Bacillus sp. T3-1 was also determined for its ability to adhere Caco-2 cells. The adhesion percentages of vegetative cells and spores were 78.3% and 74.39%, respectively. This result indicated that the vegetative cells could adhere to Caco-2 cells better than the spores did (p>0.05). In addition, preliminary screening showed that *Bacillus* sp. T3-1 had cellulase activity.

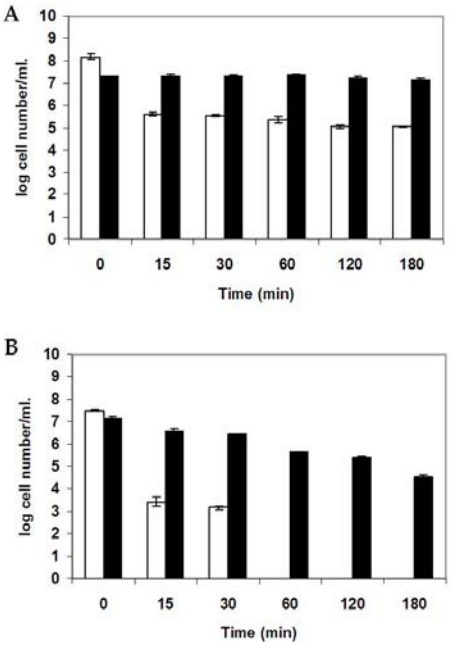

Figure 2. Heat resistance of vegetative cells (□) and spores (■) of *Bacillus* sp. T3-1 at 80°C (A) and 100°C (B). The data were the means of triplicate experiments and the error bars indicated standard error of the means.

Table 3 Resistance of *Bacillus* sp. T3-1 spores to chlorine residues at the level of 3 and 5 ppm.

Level of	% survival of spores after exposure to chlorines at different contact time[a]				
	5 min	15 min	30 min	60 min	120 min
3 ppm	98.147±2.386	97.758±1.267	98.077±1.806	97.512±1.709	97.084±1.312
5 ppm	98.763±1.689	97.282±2.284	96.873±3.333	97.060±2.662	96.113±3.062

[a]No statistical difference between each treatment was observed (p< 0.05)
The data were the means of triplicate experiments±SD of means.

Discussion

It was reported that a variety of *Bacillus* species with different probiotic characterisitics have been isolated from the gastrointestinal tract of broilers raised in 2 different English farms (Barbosa et. al., 2005). These species included *B. megaterium, B. pumilis, B. licheniformis, B. subtilis, B. cereus, B. clausii* etc. In addition, this report confirmed that origin of isolation did have effects on biological properties of isolates because 2 isolates of *Bacillus* sp. showed different profiles of probiotic properties, especially antibiotic susceptibility and antimicrobial activity spectra. In our study, 164 spore-forming isolates having different morphologies were also detected from the intestinal and cecal samples collected from chickens raised in various backyard farms. However, only *Bacillus* sp. T3-1 was selected for further characterization due to primary selection criteria including susceptibility to antibiotic, antimicrobial activity and inability to produce hemolysin.

Susceptibility test was used as the first selection criteria for safety aspect. Although percentage of isolates susceptible to each antibiotic tested were high ranging from 64 to 96%, only 38% of total isolates were susceptible to all types of antibiotic tested. The information obtained from our interview with the farmers carried out in the step of sample collection revealed that some farmers fed their chickens with antibiotics such as ampicillin, tetracycline and penicillin time to time for prophylactic or therapeutic purposes. This antibiotic abuse may be the cause of antibiotic resistance observed in our study.

Barbosa et al. (2005) demonstrated that spores of *B. subtilis* were highly resistant to stimulated gastric juice and simulated intestinal juice containing 2% bile salt whereas viability of vegetative cells decreased more than 98% within 5 min of exposure to both digestive juices. Although spores of *Bacillus* sp. T3-1 showed great resistance to both digestive juices, the vegetative cells were susceptible to only simulated intestinal juice, not simulated gastric juice. This discrepancy of the results may be attributed to higher concentration of bile salt used in our study. As known, transit time in the small intestine of chicken is about 120 min. Therefore, the amount of *Bacillus* sp. T3-1 vegetative cells left may be high enough to be able to express their metabolic functions in the small intestine.

The major factor responsible for resistance of spores to wet heat is the core water content. The higher the core water content, the less wet heat resistance of the spores (Nicholson et. al., 2000). Generally, spores are resistant to wet heat approximately 40°C higher than the vegetative cells are (Setlow, 2006). As a consequence, the spores of *Bacillus* sp. T3-1 are supposed to resist wet heat up to 120°C. However, our findings showed that *Bacillus* sp. T3-1 spores exposed to wet heat at 100°C gradually reduced with time. After 180 min of contact time, the number of spores were about 2.6 log reduced. The low wet heat resistance of spores could be explained by several reasons including lacking of dipicolinic acid (DPA) in spore formation, temperature used in spore preparation as well as the strains of spore-fomers. Paidhungat et al. (2000) demonstrated that spores of *B. subtilis* formed without dipicolinic acid (DPA) had much greater core water content than those formed with DPA. Therefore, the DPA less spores showed less resistant to the wet heat. In addition, spores prepared at lower temperatures were less resistant to wet heat than those prepared at higher temperatures. It may be possible that *Bacillus* sp. T3-1 lacked the ability to synthesize DPA. Its spores formation was carried out at 37°C in DPA-free sporulation medium. As a consequence, *Bacillus* sp. T3-1 formed spores at the low temperature without DPA resulting in low wet heat resistant spores. Although spores of *Bacillus* sp. T3-1 showed lower resistance to wet heat than expected, they can still be used without significant loss of amount in the pelleting process where the feed is exposed to heat for a few minutes.

Taken all together, the local strain of *Bacillus* sp. T3-1 isolated from backyard chicken was shown *in vitro* to have some characteristics of probiotic. On-farm experiments to determine the safety and efficiency of *Bacillus* sp. T3-1spores as feed additive for broilers are under study.

References

AL–Sultan, S.I. 2003. The effect of *Curcuma longa* (Tumeric) on overall performance of broilers chickens. Int. J. Poult. Sci. 2(5): 351–353.

Barbosa, T.M., Serra, C.R., La Ragione, R.M., Woodward, M. J. and Hariques, A.O. 2005. Screening of *Bacillus* isolates in the broiler gactrointestinal tract. Appl. Environ. Microbiol. 71 (2): 968-978.

Branda, S. S., Gonzalez-Pastor, J.E., Ben-Yehuda, E.S., Losick, R. and Kolter, R. 2001. Fruiting body formation by *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA. 98:1162-11626.

Cartman, S.T., La regione, R.M. and Woodward, M.J. 2007. Bacterial spore formers as probiotics for poultry. Food Sci. Tech. Bull. 4: 21-30.

Cartman, S.T., La regione, R.M. and Woodward, M.J. 2008. *Bacillus subtilis* spores germinate in the chicken gastrointestinal tract. Appl. Environ. Microbiol. 74(16): 5254–5258.

Clinical and Laboratory Standards Institute. Performance standards for antimicrobial disk susceptibility tests: approved standards 7th ed. Wayne, PA: Clinical and Laboratory Standards Institute, 2003. CLSI document M2-A8.

Ghazala, A.A. and Ali, A.M. 2008. Rosemary leaves as a dietary supplement for growth in broiler chickens. Int. J. Poult. Sci. 7(3): 234-239.

Hankin, L. and Anagtostakis, S. 1977. Solid media containing carboxymethyl celloluse to detect CM cellulase activity of microorganisms. J. Gen. Microbiol. 98: 109-115.

Jin, L.A., Ho, Y.W., Abdullah, N. and Jalaludin, S. 1998. Growth performance, intestinal microbial populations, and serum cholesterol of broilers fed diets containing Lactobacillus culture. Poult. Sci. 77: 1259-1263.

Meslin, F.X. 1997. Global aspects of emerging and potential zoonoses: a WHO perspective. Emerg. Infect. Dis. 3(2): 223-228.

Moroni, O., Kheadr, E., Boutin, Y., Lacroix, C. and Fliss, I. 2006. Inactivation of adhesion and invasion of food-borne *Listeria monocytogenes* by bacteriocin-producing Bifidobacterium strains of human origin. Appl. Environ. Microbiol. 72 (11): 6894-6901.

Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H. J. and Setlow, P. 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Mol. Biol. Rev. 64: 548-572.

Paidhungat, M., Setlow, B., Driks, A. and Setlow, P. 2000. Characterization of spores of *Bacillus subtilis* which lack dipicolinic acid. J. Bacteriol. 182: 5505-5512.

Patterson, J.A. and Burkolder, K.M. 2003. Application of prebiotics and probiotics in poultry production. Poult. Sci. 82: 627-631.

Pinchuk, I.V., Bressollier, P., Verneuil, B., Fenet, B., Sorokulova, I.B., Megraud, F. and Urdact, M.C. 2001. *In Vitro* Anti-*Helicobacter pylori* activity of the probiotic strain *Bacillus subtilis* 3 is due to secretion of antibiotics. Antimicrob. Agents Chemother. 45(11): 3156-3161.

Pugsley A.P. 1985. *Escherichia coli* K12 strains for use in the identification and characterization of colicins. J. Gen. Microbiol. 131: 369-376.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1999. Molecular Cloning: A laboratory Manual 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Serratosa, J., Blass, A., Rigau, B., Mongrell, B., Rigua, T., Tortades, M., Tolosa, E., Aguilar, C., Ribó, O. and Balagué, J. 2006. Residues from veterinary medical products, growth promoters and performance enhancers in food-producing animals: a European Union perspective. Rev. sci. tech. Off. Int. Epiz. 25: 637-653.

Setlow, P. 2006. Spores of *Bacillus subtilis*: Their resistance to and killing by radiation, heat and chemicals. J. Appl. Microbiol. 101: 514-525.

Teo, A.Y.L. and Tan, H.M. 2006. Effect of *Bacillus subtilis* PB6 (CloSTAT) on Broilers Infected with a pathogenic strain of *Escherichia coli*. J. Appl. Poult. Res. 15: 229-235.

Wekhe, S.N., Ogbamgba, K.O. and Oboh, C.C. 2007. Preliminary investigation of the effect of *Rhizophora racemosa* (mangrove) feed additive on broiler performance. Afr. J. Biotech. 6(16): 1963-1965.