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Protection activity of a novel probiotic strain of *Bacillus subtilis* against *Salmonella* Enteritidis infection

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ABSTRACT

The activity of 240 bacterial isolates screened from the gastrointestinal tracts of native chickens were evaluated for use as a potential probiotic in food animal production in order to protect against animal diseases and reduce pathogenic contamination of human food products. In observing the antagonistic activity of 117 bacilli isolates, 10 of these isolates exhibited higher growth inhibition of seven foodborne pathogens, including *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus, Listeria monocytogenes*, and *Vibrio cholerae*. Beneficial probiotic criteria from these isolates – which included non-pathogenicity, acid and bile salt tolerance, hydrophobicity, and adhesion to intestinal epithelial cells – exhibited that one isolate of NC11 had the most potential as a probiotic. 16S rRNA gene sequencing showed that this NC11 isolate was *Bacillus subtilis*. This *B. subtilis* NC11 was sensitive to all antibiotics and was not cytotoxic to intestinal epithelial cells. Reduction of *S.* Enteritidis attachment to the surfaces of intestinal epithelial cells via action of a cultured medium from *B. subtilis* NC11 was observed by scanning electron microscopy. *B. subtilis* NC11 cells, as well as the bacterial cultured medium or the cultured medium adjusted to pH 7, significantly inhibited *S.* Enteritidis invasion ($P < 0.01$) of intestinal epithelial cells. This study indicates that *B. subtilis* NC11 has characteristics of a potential probiotic, and exhibits strong inhibition activity against *S.* Enteritidis infection to intestinal epithelial cells.

1. Introduction

Salmonella is a major foodborne pathogen that is found in poultry products and can cause severe illness in humans. Diseases and syndromes such as enteric fever, bacteremia, focal infection, and enterocolitis are caused by this type of pathogenic bacterium. Human salmonellosis has become an important international public health and economic issue (Duguid and North, 1991; Velge et al., 2005). Human health protection through the elimination of foodborne pathogens from food animals and their products has been an increasing concern for all sectors of the food production chain (La Ragione and Woodward, 2003; La Ragione et al., 2001). The widespread use of antibiotics as therapeutic agents and growth promoters in animal husbandry has led to a worldwide increase in the antibiotic resistance of bacteria, an imbalance of normal microflora, and drug residues in food products. Consequently these compounds have been banned as animal feed additives by the European Union (Perreten, 2003). One alternative method that has been recommended due to its successful application is the use of probiotics (Reuter, 2001). Our previous study (Samanya and Yamauchi, 2002) suggested that due to its probiotic potential, *Bacillus subtilis* var. *natto* is one strain that could be promoted as a biological product intended for humans or animals.

There are many criteria that must be investigated before establishing that a new strain of bacteria is a probiotic. These criteria must include the non-pathogenicity of the microorganism, its ability to inhibit the growth of harmful organisms, its tolerance for acid and bile salt conditions, and its ability to adhere to intestinal epithelial cells (Salminen et al., 1998). Competitive exclusion is one of the modes of action of a beneficial probiotic that is exhibited to protect against infection from any pathogen in the intestinal epithelial cells of animal host. However, it is unclear whether the effective action of competitive exclusion by using probiotic *Bacillus* spp. will result in solo or combined actions in the gastrointestinal tract of the host, including immunomodulation, competition for adhesion sites, and production of antimicrobial agents (Patterson and Burkholder, 2003). Lactic acid bacteria (LAB) have been found to inhibit infection by *Salmonella* spp. to the intestinal epithelial cells (Tsai et al., 2005; Golowczyc et al., 2007). There was no report, however, of any effective action of probiotic *Bacillus* spp. in inhibiting the infection of *Salmonella* in the intestinal epithelial cells.

The aims of this study were to investigate the activity of isolates of *Bacillus* spp. for possible use as potential probiotics, and

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their protective inhibition activity against *Salmonella* Enteritidis infection.

2. Materials and methods

2.1. Bacterial isolation

Thirty-three native chickens (12–24 weeks old, weight 1–2 kg) were collected. They had been raised under natural farming and feeding conditions in different areas of Thailand. All animals were handled in accordance with institutional guidelines for the care and use of animals in research, and in compliance with the ethical principles and guidelines for the use of animals for scientific purposes of the National Research Council of Thailand. Fresh intestinal content weighing 1 g, obtained after dissection of the chicken small intestine, was collected from each chicken and then tenfold diluted with 0.85% NaCl in sterilized distilled water. Suitable dilutions of 100 μl were spread on nutrient agar (NA; Merck, Darmstadt, Germany) and incubated at 37 °C in aerobic conditions for 24 h. Morphologically different colonies, about 6–9 colonies per chicken, were collected. In order to obtain pure isolation, restreaking on agar of the same media was done. A total of 240 bacterial isolates were obtained. Each isolate was stored at −80 °C in nutrient broth (NB; Merck), supplemented with 20% glycerol (Merck) until further study.

2.2. Bacterial morphology and catalase assay

The fundamental morphological determination of *Bacillus* genera – Gram and spore-staining – was performed as previously described by Benson (1998). Gram-staining of each isolate was done in order to separate Gram-positive, Gram-negative, rod, and cocci forms. Only isolates that were Gram-positive and rod shaped were further examined by Schaeffer-Fulton staining method to confirm that the isolates were endospore-forming after bacterial culture for 24 h. For the catalase assay, the method of Barbosa et al. (2005) was used; bacterial isolates which were grown in NB were incubated at 37 °C for 18 h. The resuspension of a colony in a 3% solution of hydrogen peroxide was evaluated. The isolates exhibited the formation of gas bubbles, indicating a positive test (catalase-positive). This assay was done in duplicate.

2.3. Antagonistic activity of bacilli isolates against foodborne pathogens

A total of 117 bacilli isolates – Gram-positive rods, endospore-forming and catalase-positive – were further studied for their effect against foodborne pathogens including *S.* Enteritidis DMST 15676, *S.* Typhimurium TISTR 292, *Escherichia coli* TISTR 780, *Bacillus cereus* TISTR 687, *Staphylococcus aureus* TISTR 118, *Listeria monocytogenes* DMST 1783, and *Vibrio cholerae* DMST 2873. A modification of the paper disc (Durchmesser: 6 mm; Macherey–Nagel, Duren, Germany) diffusion method was used for triplicate tests, as previously suggested by Sansawat and Thirabunyanon (2009). Pathogenic and tested bacteria were grown in NB, and incubated at 37 °C for 18 h in aerobic conditions at an adjusted approximate concentration of 10^8 CFU/ml. The only exception was pathogenic *L. monocytogenes*, for which brain heart infusion (Criterion, Santa Maria, USA) was used. This was incubated at 37 °C for 18 h in anaerobic conditions. Sterilized paper discs were impregnated with 20 μl of diluted tested bacterial isolates and placed on the surface of agar plates which were already inoculated with indicator pathogens at a concentration of about 10^7 CFU. Next, the plates were incubated at 37 °C for 24 h, and inhibition zones around the paper discs were recorded.

2.4. Acid and bile salt tolerance assay

Acid tolerance was evaluated by a modified method of Pennacchia et al. (2004). Bacterial cultures were grown in NB at 37 °C for 18 h. One milliliter of bacterial suspension was then transferred into 9 ml of phosphate buffered saline (PBS) with pH value adjusted to 2.5 using 5 M HCl (Merck). The suspensions were again incubated at 37 °C and later examined for survival rate after exposure at 0 and 3 h. The number of viable counts was determined after incubation at 37 °C for 24 h on NA. This method was done in triplicate. For bile salt tolerance, the methods of Gilliland et al. (1984) and Pennacchia et al. (2004) were performed. One milliliter of bacteria cultured for 18 h was inoculated into 9 ml of NB prepared with bile salt (Sigma, St Louis, MO, USA). Bile salt at a concentration of 0.3% was applied. The suspension was incubated at 37 °C and the viable bacteria were counted after exposure at 0 and 24 h on NA. This method was performed in triplicate.

2.5. Hydrophobicity assay

The method according to Savage (1992) was used. Bacterial isolates were grown in NB at 37 °C for 18 h. After centrifugation at 5000*g* for 15 min, the pellets were washed twice with PBS and the absorbance at 450 nm adjusted to 0.5 with an approximate concentration of 10^7 CFU/ml. About 1 ml of bacterial suspension was added to 60 μl of hydrocarbons *n*-hexadecane (Fluka, Buchs, Germany), xylene (Fisher, Loughborough, England), and toluene (Merck), and vortexed for 1 min. The suspensions were left undisturbed for 1 h, and the OD of the water phase was measured. The method was conducted as duplicate. Hydrophobicity was calculated according to the equation: $[(OD_{450}\text{ before} - OD_{450}\text{ after}/OD_{450}\text{ before} \times 100) = \%\text{ hydrophobicity}]$.

2.6. Cell culture

Cells of the intestinal epithelial cell line (Caco-2) were routinely grown in Dulbecco's modified Eagle's minimal essential medium (DMEM; Sigma–Aldrich, USA) supplemented with 10% fetal calf serum (Hyclone, Logan, Utah, USA) inactivated at 56 °C for 30 min, 1% (v/v) non-essential amino acid (Hyclone), and 1% (v/v) penicillin–streptomycin (10,000 IU/ml and 10,000 μg/ml; Hyclone). Cells were incubated at 37 °C in 5% CO_2 atmosphere. For bacilli adhesion, and *Salmonella* attachment or invasion assays, intestinal epithelial cells were seeded with 1 ml of culture medium containing 10^6 viable cells/well in 24-well tissue culture plates. The culture medium was changed every 48 h. The intestinal epithelial cells were used at 15 days post-confluence after becoming fully differentiated. The medium of non-supplemented DMEM was replaced at least 1 h before these assays.

2.7. Adhesion of bacilli isolates to intestinal epithelial cells

Testing of the ability of bacilli to adhere to intestinal epithelial cell culture monolayers was performed in 24-well tissue plates, as previously described by Gagnon et al. (2004) and Bogovic Matijasić et al. (2006). Bacilli isolates from 18 h cultures in NB were harvested by centrifugation and washed twice with PBS. These bacilli isolates were then resuspended in non-supplemented DMEM to achieve a concentration of 10^8 CFU/ml. After washing the intestinal epithelial cell culture monolayer twice with PBS, 0.5 ml of bacilli suspension was added to each well and then incubated at 37 °C for 1 h in 5% CO_2 in air. Removing unattached bacteria was performed by washing with PBS three times while intestinal epithelial cells were lysed with 0.1% Triton X-100 (Merck) for 5 min. Viability of adherent bacilli was done by plate counting in triplicates on NA. This analysis was done in five replicate.