Biocontrol of the Internalization of *Salmonella enterica* and Enterohaemorrhagic *Escherichia coli* in Mung Bean Sprouts with an Endophytic *Bacillus subtilis*

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Abstract

Internalization of *Salmonella enterica* and enterohaemorrhagic *Escherichia coli* (EHEC) in seed sprouts poses a health risk to consumers, and the conventional sanitization methods are not always effective to reduce this risk. This study initiated a biocontrol approach to limit the internalization using endophytic *Bacillus subtilis* strains, which were isolated from the inner tissue of mung bean seeds or lettuce stems. By using the deferred agar method, 12 strains of *B. subtilis* out of 94 putative *Bacillus* isolates displayed inhibitory activity against at least one of the pathogenic indicators, *S. enterica* Typhimurium ATCC 14028 and *E. coli* O157:H7 505B. Two *B. subtilis* isolates (LCA1 and M24) showed a broad inhibitory spectrum against multiple strains of *S. enterica* and EHEC, *Staphylococcus aureus* sp., *Klebsiella pneumoniae* ATCC 700603, and *Listeria monocytogenes* Scott A, while the laboratory *B. subtilis* strain 168 was only moderately inhibitory against *L. monocytogenes*. To facilitate the tracking of the three *B. subtilis* strains (LCA1, M24, and 168) in the mung bean sprouts, the three strains were genetically engineered to carry the chloramphenicol acetyltransferase (*cat*), generating the strains LCA1-*cat*, M24-*cat*, and 168-*cat*, respectively. Data of the study using the *cat*-tagged strains demonstrated that both the two vegetable-associated and the laboratory *B. subtilis* strains could internalize in mung bean sprouts during the sprouting, but the latter displayed about 1.2 lg CFU/g of seeds lower in internalization. Overall, the presence of the three *B. subtilis* strains could significantly reduce the internalization of *S. enterica* or EHEC cocktail in mung bean sprouts during the sprouting. Among them, LCA1 showed the greatest inhibition against the EHEC cocktails with a reduction of about 2.0 lg CFU/g of seeds by the end of sprouting (day 5), while 168 had the smallest reduction at about 0.6 lg CFU/g of seeds. In addition, the three strains demonstrated a similar inhibition against the *S. enterica* cocktails by a reduction of about 1.1~1.4 lg CFU/g of seeds by

day 5. Results of this study suggest that the source (native vs. alien) of *B. subtilis* isolates may not affect the efficacy of the inhibition, but it might be affected by the production of antimicrobial substance and/or nutrition/space competition. The results also indicate that strain LCA1 may be useful as a biocontrol agent to reduce *Salmonella* and EHEC contamination in seed sprouts.

Keywords: food safety, fresh produce, foodborne illness, antagonistic

1. Introduction

Seed sprouts have been associated with several recent foodborne disease outbreaks (Yang et al., 2013). With the increasing consumption of fresh vegetables as a part of a healthy human diet, seed sprouts have emerged as a serious challenge in food safety because they are usually consumed raw or with little processing, in addition to being produced in warm and humid conditions that are ideal for pathogens to grow (Fu et al., 2001; Taormina et al., 1999). Sprouts involved in recent outbreaks were alfalfa, clover, fenugreek, and mung bean, while the associated pathogens included multiple serotypes of *Salmonella enterica* and several enterohaemorrhagic *Escherichia coli* (EHEC) (Fu et al., 2001; Radosavljević et al., 2015; Radosavljević et al., 2016; Sivapalasingam et al., 2004; Taormina et al., 1999; US FDA. 2001; Watanabe et al., 1999; Winthrop et al., 2003; Yang et al., 2013).. Among all produce-associated foodborne disease outbreaks, sprout- *Salmonella* has been identified as the number one carrier-pathogen combination (deWaal, 2007). The 2011 massive outbreak caused by fenugreek sprouts contaminated with enterohaemorrhagic *E. coli* O104:H4 in Europe, killing more than 50 people, once again increased the critical public health concerns about sprout safety (Denamur, 2011: Radosavljević et al., 2015 and 2016).

To control disease outbreaks associated with seed sprouts, the U.S. Food and Drug Administration (US FDA) in 1999, issued guidelines recommending all seeds for sprouting be decontaminated with 20,000 ppm of calcium hypochlorite for at least 15 min prior to sprouting (US FDA, 1999). However, this method has been proved not to be fully effective for at least two reasons (Fett, 2002a; Proctor et al., 2001; Singh et al., 2005). First, it is a one-time control approach. Once the control is removed, any residual pathogens surviving from the decontamination can rapidly grow to high levels, posing new risks to human health (Holliday et

al., 2001). Second, it has been reported that *E. coli* O157:H7 and several serotypes of *Salmonella* could internalize in and reside in sprout inner tissues (Deering et al., 2011; Dong et al., 2003a; Dong et al., 2003b; Gandhi et al., 2001; Gu et al. 2011 and 2013a; Itoh et al., 1998; Warriner et al., 2003). The internalized pathogens in seed sprouts are a serious food safety challenge as they are protected by sprout tissues from surface sanitization.

Many chemical and physical approaches have been proposed as alternative interventions to control *Salmonella* spp. and *E. coli* O157:H7 contamination in seed sprouts, including immersing seeds in chlorine (Hora et al., 2007), organic acids (Delaquis et al., 1999; Zhao et al., 2010), hydrogen peroxide (Beuchat, 1997; Fett, 2002b), ammonia (Himathongkham et al., 2001), ethanol (Beuchat, 1997), hot water (Hu et al., 2004), or treating seeds with dry heat combined with irradiation (Bari et al., 2009) or with high hydrostatic pressure (HHP) (Neetoo and Chen, 2010). Among these control procedures, only irradiation and HHP might be effective against internalized pathogens, but both methods are one-time control approaches. Furthermore, public acceptance of irradiated food remains unfavorable and chemical and/or hot water treatments have the same drawbacks as the U.S. FDA's calcium hypochlorite method (i.e., not being able to effectively reduce the internalized pathogens).

Recently, biocontrol methods using competitive or antimicrobial-producing bacteria to control foodborne pathogens in sprouts have gained increasing attention (Gu et al., 2013b; Klerk et al. 2007). The reported biocontrol agents included lactic acid bacteria (Palmai and Buchanan, 2002; Wilderdyke et al., 2004), *Pseudomonas fluorescens* (Fett, 2002b; Matos and Garland, 2005), *Enterobacter asburiae* (Ye et al., 2010), and even native microflora obtained from market sprouts (Matos and Garland, 2005). Compared with the one-time control chemical or physical methods, the biocontrol approaches can provide continuing protection during sprout production.

However, it is unclear if the biocontrol agents can be used to control the internalization of human pathogens into sprouting seeds.

Bacillus subtilis is believed to be one of the most promising biocontrol agents. First, it can produce a broad spectrum of antimicrobial substances that kill pathogenic bacteria and/or fungi (Abriouel et al., 2011; Stein, 2005; Todorova and Kozhuharova, 2010). Second, it is not known to be pathogenic to humans (Teo and Tan, 2005). Third, it forms endospores, which are resistant to harsh environmental factors, making them favorable biocontrol agents for their advantages in production, transportation, and storage in various environments without losing their viability (Driks, 2004). Furthermore, *B. subtilis* is one of the most frequently isolated bacteria from plants (Bacon, W.C. and White J., 2000). Strains of *B. subtilis* have been studied as biocontrol agents against plant diseases, such as black pod rot of cacao (Melnick et al., 2008) and *Cercospora* leaf spot in sugar beets (Bargabus et al., 2002, 2004). However, there is little research on using *B. subtilis* to control foodborne pathogens in vegetables.

The objective of this study was to investigate the potentials of native and alien strains of endophytic *B. subtilis* antagonists, isolated from the inner tissues of vegetables, in controlling the internalization of *Salmonella* and EHEC during mung bean sprouting.

2. Materials and methods

2.1. Bacterial strains, plasmids, and media

The laboratory strain *B. subtilis* 168 and the *E. coli*-*Bacillus* shuttle vector pAD43-25 were obtained from the Bacillus Genetic Stock Center (BGSC), Columbus, Ohio. The other *Bacillus* strains were isolated from the inner tissue of mung bean seeds or lettuce, as detailed in section 2.2. The pathogenic strains (Table 1) used in this study were from the stocks of Dr.

Mustapha's laboratory at the University of Missouri, Columbia, Missouri. All strains of *S. enterica*, EHEC, *Staphylococcus aureus* sp., and *Klebsiella pneumoniae* ATCC 700603 were erythromycin resistant, and the *Listeria monocytogenes* Scott A was unaffected by kanamycin. All bacterial strains were frozen at -70°C in 25% (v/v) glycerol (Sigma, St. Louis, MO) for long-term storage. When used, the bacteria were grown in lysogeny broth (LB) (Thermo Fisher Scientific Inc., Hampton, NH) at 37°C with agitation (200 rpm), unless otherwise stated. Agar plates of Difco sporulation medium (DSM) (Harwood and Cutting, 1990) were used to demonstrate the *Bacillus* isolates' antimicrobial activity.

2.2. Isolation of endospore-forming endophytes (EFEs) from vegetables

Vegetable-associated EFEs were isolated from romaine lettuce ($n = 5$), iceberg lettuce ($n = 5$), and mung bean seeds (40-g batch, $n = 3$) purchased from local supermarkets. Lettuce stems were washed thoroughly with running water, peeled with flame-sterilized knives, and then sliced into small pieces. Ten g of the lettuce stem pieces were stomached in 10 ml of sterile 0.1% peptone water (w/v) at 270 rpm for 2 min with a stomacher blender 400 (Seward, UK). Using the procedure of Warriner et al. (2003), mung bean seeds were sanitized by washing them thoroughly with running water, submerging them in 100 ml of 20,000 ppm sodium hypochlorite-100 mM-phosphate buffered saline (PBS) solution for 15 min, and finally rinsing them with 100 ml of sterile, distilled water five times. The surface-sanitized mung bean seeds were softened by soaking in 40 ml of sterile distilled water overnight at room temperature before being stomached at 270 rpm for 2 min. To isolate the EFE, the homogenates of the lettuce stems or mung bean seeds were incubated at 70°C for 30 min in a water bath to kill anything that was not EFE. One ml of each of the homogenates was spread onto 10 DSM agar plates (0.1 ml per plate) and incubated at 37°C for 48 h. Distinct *Bacillus*-like colonies (white or light yellow, irregularly

shaped, and dry or pasty looking) that appeared on the plates were randomly selected and re-streaked onto new DSM plates to obtain pure cultures. The purified isolates were grown in LB overnight and then stored at -70°C with glycerol for later use.

2.3. Screening of antagonists

With the deferred agar spot method (Fett, 2006), the putative *Bacillus* isolates were screened for antimicrobial activity against two pathogen indicators, *S. enterica* Typhimurium ATCC 14028 and *E. coli* O157:H7 505B. Ten μl of overnight culture of each isolate or *B. subtilis* 168 was spotted onto a DSM agar plate and incubated at 37°C for 48 h. Each plate was then overlaid with 8 ml of 0.7% (w/v) soft LB agar containing 50 μl of overnight culture of one of the pathogen indicators, supplemented with erythromycin at the final concentration of 50 mg/ml. After the soft agar had solidified, the plates were turned upside down and incubated at 37°C for another 48 h to observe the presence or absence of inhibition zones.

2.4. Identification of anti-pathogen *B. subtilis* isolates

The putative *Bacillus* isolates that showed significant antimicrobial activity against at least one of the pathogenic indicators were subjected to taxonomic identification by 16S rRNA gene DNA sequencing analysis followed by biochemical tests using the BioLog MicroStation™ System (Hayward, CA).

To conduct the 16S rRNA gene sequencing, genomic DNA of the isolates was extracted from 1 ml of the overnight LB cultures, using PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). The 16S rRNA gene of each isolate was amplified using polymerase chain reaction (PCR) employing the universal 16S rDNA PCR primers, i.e., Bac27F (5'-AGAGTTTGATCCTGGCTCAG-3') and Bac1392R (5'-GACGGGCGGTGTGTA-3') (Stackebrandt and Goodfellow 1991). Each 50- μl PCR cocktail contained 20 μl of 2.5 $\times$ Taq

MasterMix (5 Prime, Gaithersburg, MD), 50 pmol of each primer (Integrated DNA Technologies, Coralville, IA), and 10 ng of genomic DNA. PCR amplifications were conducted with 40 cycles of the following thermocycle profile: initial denaturation at 95 °C for 2 min; denaturation at 95 °C for 30 sec; annealing at 55 °C for 30 sec; elongation at 72 °C for 90 sec, and final elongation at 72 °C for 6 min. The resulting amplicons were purified with the GelElute Extraction Kit (5 PRIME, Gaithersburg, MD) and sequenced by the DNA Core Facility at the University of Missouri, Columbia, Missouri. The 16S rRNA gene sequences thus obtained were subjected to taxonomic classification using the Classifier program provided by the Ribosomal Database Project 10 (RDP) (Cole et al., 2009). The taxonomic identification of each antagonist was further confirmed by phenotypical identification using the Biolog MicroStation™ System, following the manufacturer's procedure. Anti-pathogen isolates thus classified as *B. subtilis* were used for this study.

2.5. Construction of *cat*-tagged *B. subtilis* antagonists

To facilitate their tracking in plant tissues, anti-pathogen *B. subtilis* were tagged with the chloramphenicol-resistant-gene(*cat*)-carrying plasmid, pAD43-25, by following the electroporation protocol described by Xue et al. (1999), with modifications. Briefly, the *B. subtilis* strains were grown in 50 ml of LB containing 0.5 M sorbitol in the later exponential phases (OD₆₀₀ 0.85~0.95). Cells were harvested by centrifugation at 4 °C and 5000 ×g for 5 min, and washed four times with ice-cold electroporation medium (0.5 M sorbitol, 0.5 M mannitol, 10% glycerol). Cell pellets were then suspended in 1 ml of the electroporation medium. Each 1 µl of (100 ng/µl) pAD45-25 DNA was added to 60 µl of cells and gently mixed. The cell-DNA mixture was electroporated in a prechilled electroporation cuvette (1 mm gap width) with an electroporator 2590 (Eppendorf, Hamburg, Germany) at 2,100 V. The electroporated cells were

transferred to 1 ml of LB containing 0.5 M sorbitol and 0.38 M mannitol and incubated for 3 h at 37°C. Each 0.1 ml of the electroporated cell cultures was spread onto LB plates supplemented with chloramphenicol at 5 mg/ml. To obtain pure cultures, several transformant colonies were streaked on new LB agar plates containing chloramphenicol. The method successfully generated *cat*-tagged *B. subtilis* strains from isolates LCA1 and M24 as well as from laboratory strain 168, but not from the other isolates. The derived strains were designated LCA1-*cat*, M24-*cat*, and 168-*cat*, respectively. Plasmid pAD45-25 was relatively stable in the derived strains, being able to maintain in the cells for at least 10 generations at the absence of chloramphenicol (data not shown) and adequate for our experiments.

2.6. Antimicrobial spectra of the *cat*-tagged derivatives on agar

Antimicrobial spectra of strains LCA1-*cat*, M24-*cat*, and 168-*cat* were determined with the deferred agar spot method, as described in section 2.3, using the pathogens, including multiple strains of *S. enterica* and EHEC, *S. aureus* sp., *K. pneumoniae* 700603, and *L. monocytogenes* Scott A (Table 1). The overlaid soft agar was supplemented with 50 mg/ml of erythromycin when *S. enterica*, EHEC, *S. aureus* sp., and *K. pneumoniae* 700603 were used, or with 5 mg/ml of kanamycin when *L. monocytogenes* Scott A was used. The radii of the inhibition zones were measured after 48 h incubation at 37°C. All plates were prepared in duplicate and the experiment was repeated three times.

2.7. Inhibition of the *cat*-tagged *B. subtilis* against *S. enterica* and EHEC in LB

LCA1-*cat*, M24-*cat*, and 168-*cat* were tested for their inhibitory activity against the growth of the cocktails of *S. enterica* and EHEC in LB by adapting the protocol described by Fett (2006), with slight modifications. Briefly, the 10 strains each of *S. enterica* and EHEC listed in Table 1 were used for this experiment. The *cat*-tagged *B. subtilis* strains and the pathogens

were grown individually in LB at 37 °C with agitation (200 rpm/min) overnight. The cells of the bacteria were harvested by centrifugation (5,000 ×g, 15 min), washed once with sterile 0.1% peptone water, and adjusted with the peptone water to an OD₆₀₀ of 0.01. Equal volumes of the 10 strains of *S. enterica* or EHEC suspensions were mixed to make *S. enterica* and EHEC cocktails, respectively. The cocktails were then diluted 10,000 fold with sterile 0.1% peptone water. Two ml of each *cat*-tagged *B. subtilis* suspension was co-inoculated with 2 ml of the diluted *S. enterica* or EHEC cocktail in a 250-ml flask containing 16 ml of LB, to create initial cell densities of the *B. subtilis* and the pathogen cocktails of approximately 6 and 3 lg CFU/ml, respectively. A single inoculation of the *S. enterica* cocktail, EHEC cocktail, LCA1-*cat*, M24-*cat*, or 168-*cat* was set up as the controls. All cultures were grown at 37 °C with agitation, and 1 ml aliquots were withdrawn from each culture at 0, 4, and 24 h. The samples were serially diluted with 0.1% peptone water and plated in duplicate onto LB agar plates supplemented with erythromycin (50 mg/ml, for the pathogens) or chloramphenicol (5 mg/ml, for the *B. subtilis*). The plates were incubated at 37 °C for 24 h, and then the colonies appearing on the plates were counted. The experiment was conducted for three times.

2.8. Inhibition by internalized *B. subtilis* against *S. enterica* or EHEC cocktails in sprouting mung beans

Overnight cultures of LCA1-*cat*, M24-*cat*, and 168-*cat* were inoculated 1:100 in fresh LB, and the cells were harvested at OD₆₀₀ of 0.4~0.6, washed with sterile PBS solution three times by centrifugation (5,000 ×g, 15 min), and then suspended in PBS to OD₆₀₀ of 0.2. With the same procedure, the 10 *S. enterica* and 10 EHEC strains (Table 1) were grown, harvested, washed, and then suspended in PBS. Equal volumes of the 10 *S. enterica* and 10 EHEC PBS suspensions were used to make *S. enterica* and EHEC cocktails, respectively.

On day 0, 120 g of mung bean seeds with intact skin were washed thoroughly with running water, submerged in 200 ml of 20,000 ppm sodium hypochlorite PBS for 15 min for surface disinfection, rinsed with 200 ml of sterile water five times, and then dried on sterile filter paper for 2 h. The mung bean seeds equally divided into four groups (30 g/group) were separately placed in a sterile beaker and submerged for 2 h in 120 ml of PBS (the control), the LCA1-*cat*, M24-*cat*, or 168-*cat* suspensions, respectively. The seeds of each group were then dried on sterile filter papers for 2 h and placed respectively in three sterile Falcon 50-mL tubes for use the next day.

On day 1, seeds of each group were further equally divided into three subgroups (10 g/group) and placed respectively in three new sterile Falcon 50-mL tubes. Forty ml of PBS solution (the control), the cocktail of *S. enterica*, or the cocktail of EHEC, was respectively added to the three tubes to cover the seeds. After 2 h, the seeds of each subgroup were again dried on sterile filter papers for 2 h and then separately placed on the top of sterile papers saturated with sterile distilled water in three sterile 500-ml glass beakers. The beakers were kept at room temperature (22 °C) in the dark for five days for the seeds to sprout. The seeds (day 1) or sprouts (days 2 to 5) were watered twice daily in the morning and evening. This was done by adding 200 ml of sterile distilled water, gently shaking the beaker with hands, and then carefully dumping the excess water while keeping the seeds and sprouts in the beaker with a pair of sterile forceps. On days 2, 3, 4, and 5, ten sprouts were withdrawn from each beaker for enumeration of internalized *B. subtilis* and pathogens.

To enumerate the internalized *B. subtilis* and pathogens, seeds or sprouts were washed three times, submerged in 40 ml of 20,000 ppm sodium hypochlorite-PBS for 15 min for surface disinfection, and then rinsed five times with 40 ml sterile distilled water to remove any sanitizer

residues. The surface-sanitized seeds or sprouts were then manually ground in stomach bags with 10 ml of sterile 0.1% peptone water. The homogenates were serially decimal-diluted with sterile 0.1% peptone water, and 0.1 ml aliquots of undiluted or diluted homogenates were plated onto LB agar plates supplemented with erythromycin (50 mg/ml, for pathogens) or chloramphenicol (5 mg/ml, for *B. subtilis*). The last rinsing from each treatment was examined for the surface sanitization effect using the pour-plate protocol (Sanders, 2011). A 1 ml aliquot of the last rinsing water was added to an empty petri dish, and 20 ml of molten LB agar containing erythromycin (for pathogens) or chloramphenicol (for *B. subtilis*) was then added. In total, 10 ml of each last rinse was tested. The plates were gently swirled and left on the bench for agar solidification. All plates were reversed and incubated at 37°C. Bacterial colonies were counted after 24 h of incubation.

All plates were prepared in duplicate, and the experiment was repeated three times. . The CFU per g of seed was calculated and statistically analyzed from the results of the triplicates.

2.9. Statistical analysis

Inhibitory effects of the *B. subtilis* strains on the *S. enterica* or EHEC cocktail in the LB broth was analyzed with the one-way ANOVA followed by Bonferroni post hoc tests to determine if there was a significant inhibitory difference ($P < 0.05$) between any two *B. subtilis* strains at each sampling time point.

The two-way ANOVA was used to statistically analyze the data of *in planta* tests to determine for each sampling time if there was a significant difference ($P < 0.05$) among the populations of the *B. subtilis* strains (the first factor) or those of the pathogen cocktails (the second factor), and if there was a significant interaction between the two factors. The populations of the two factors were converted into lg CFU per g seed prior to the statistical analysis. In the

analysis, the first factor was composed of 4 levels (the control, LCA1-*cat*, M24-*cat*, and 168-*cat*), while the second factor 3 levels (the control, *S. enterica*, and EHEC). One-way ANOVA was then used to analyze if there was a significant effect ($P < 0.05$) of a factor on the other factor at each sampling time. Finally, a Bonferroni post hoc test was used to determine if there was a significant change ($P < 0.05$) between any two levels of a factor at each sampling time.

3. Results

3.1. *B. subtilis* antagonists isolated from vegetables

A total of 94 EFE isolates were obtained from the inner tissues of the lettuce stems (74 isolates) and mung bean seeds (20 isolates). By the deferred agar spot method, 12 isolates were determined to be active against either *S. enterica* Typhimurium ATCC 14028 or *E. coli* O157:H7 505B (Table S1). No inhibition was observed for the laboratory strain 168. All of the 12 anti-pathogen isolates were identified as *B. subtilis* by the 16S rRNA gene sequencing analysis, followed by using the Biolog® Microbial Identification System.

3.2. *In vitro* antimicrobial tests of the *cat*-tagged *B. subtilis*

As summarized in Table 1, on the agar plates, both LCA1-*cat* and M24-*cat* demonstrated inhibitory activities against all Gram-positive and -negative pathogens tested, while the *cat*-tagged laboratory strain, 168-*cat*, showed a moderate inhibition only against Gram-positive *L. monocytogenes* Scott A. Generally, LCA1-*cat* had stronger inhibitory activities than M24-*cat*. LCA1-*cat* formed clear inhibition zones against most of the pathogens, while about half of the inhibition zones formed by M24-*cat* were turbid. All the inhibition zones generated by LCA1-*cat* were larger than those generated by M24-*cat*, except when *S. aureus*, *S. enterica* Rubislaw, or *S. enterica* Typhimurium ATCC13311 were used.

As shown in Fig. 1, the *S. enterica* and EHEC cocktails reached approximately 5.0 lg CFU/ml in 4 h and about 9.0 lg CFU/ml in 24 h in LB (Fig. 1A, and B), after an initial concentration of 3.0 lg CFU/ml. When co-inoculated with 6 lg CFU/ml of *B. subtilis* 168-*cat*, LCA1-*cat*, or M24-*cat*, an inhibitory effect was not observed at 4 h against either *S. enterica* or EHEC. By 24 h, *S. enterica* concentrations were 7.3, 7.4, and 7.1 lg CFU/ml in the presence of LCA1-*cat*, M24-*cat*, or 168-*cat*, respectively, showing a significant reduction by more than 1.0 lg CFU/ml when compared with the control (Fig. 1A). Similarly, by 24 h, the EHEC concentrations were 6.9, 6.9, and 7.0 lg CFU/ml, respectively, demonstrating a significant reduction of more than 1.5 lg CFU/ml (Fig. 1B). In contrast, the concentrations of LCA1-*cat*, M24-*cat*, and 168-*cat* were not affected by the presence of either *S. enterica* or EHEC by both 4 h and 24 h (data not shown). In general, the three *Bacillus* strains showed no significant difference in inhibition of either *Salmonella* or *E. coli* in broth, contradictory to what was observed on the agar plates (Table 1).

3.3. Internalization of the cat-tagged B. subtilis, S. enterica, and EHEC in mung bean sprouts during the sprouting

The results of the enumeration of the chloramphenicol-resistant bacteria from the surface-sanitized, pathogen-free sprouts showed that the three *cat*-tagged *B. subtilis* strains could internalize in the mung bean sprouts no later than day 2 of sprouting and could maintain a relatively stable concentration over the whole sprouting period (Fig. 2A). The concentrations of LCA1-*cat* and M24-*cat* were at about 4.8 lg CFU/g during the entire sprouting period, while those of 168-*cat* were about 1.2 lg CFU/g less at all times ($P < 0.05$).

In the *Bacillus*-free sprouts, similar amounts of *S. enterica* cocktail (6.4 lg CFU/g) and EHEC cocktail (6.8 lg CFU/g) appeared to internalize in sprouts ($P > 0.05$) (Fig. 2B) by day 2.

However, the numbers of both internalized pathogens reduced at day 4 (5.3 and 6.4 lg CFU/g respectively), and further reduced at day 5 (3.9 and 5.1 lg CFU/g respectively). The reduction of *S. enterica* was significantly greater than that of EHEC at day 4 and 5 ($P < 0.05$).

3.4. Effects of the internalized *S. enterica* or EHEC on *B. subtilis* internalization

Concentrations of the internalized LCA1-*cat*, M24-*cat*, and 168-*cat* in mung bean sprouts were not affected by the presence of either internalized *S. enterica* or EHEC ($P > 0.05$) at all sampling time points (Fig. 3A, B, and C).

3.5. Effects of the internalized *B. subtilis* against the internalization of the *S. enterica* or EHEC

Compared with the *Bacillus*-free control, internalization of the *S. enterica* cocktail in mung beans during sprouting was significantly inhibited by the internalized LCA1-*cat*, M24-*cat*, or 168-*cat*, but this occurred at various levels (Fig. 4). On day 2, LCA1-*cat* showed a significantly greater inhibition against the internalization of *S. enterica* than did 168-*cat*; however, on the later sampling days, all three strains displayed a similar inhibition ($P > 0.05$). The greatest reduction, 1.6 lg CFU/g, was observed with the co-inoculation of LCA1-*cat* and *S. enterica* on days 2.

Similarly, all three *cat*-tagged *B. subtilis* strains could inhibit the internalization of the EHEC cocktail in mung bean sprouts (Fig. 4B). Among the three strains, there was no significant difference ($P > 0.05$) in inhibition against the EHEC cocktail before day 4. On day 4, a significant difference ($P < 0.05$) in inhibition activities was observed, ranked from the greatest to the least difference: LCA1-*cat*, M24-*cat*, and 168-*cat*. On day 5, the inhibition activity of LCA1-*cat* was significantly higher than that of M24-*cat* or 168-*cat*.

Overall, the presence of the three *B. subtilis* strains could significantly reduce the internalization of *S. enterica* or EHEC cocktail in mung bean sprouts. By the end of sprouting

(day 5), among the three *B. subtilis* strains LCA1 showed the greatest inhibition against the EHEC cocktails, with a reduction of about 2.0 lg CFU/g, while 168 had the smallest reduction, at about 0.6 lg CFU/g. In contrast, the three strains demonstrated a similar inhibition against the *S. enterica* cocktails by a reduction of 1.1~1.4 lg CFU/g by day 5.

4. Discussion

This study was conducted to explore the potential of endophytic *B. subtilis* as useful biocontrol agents for reducing the food safety risk of fresh produce. Our data suggest that the anti-pathogen *B. subtilis* can be used in controlling *Salmonella* and pathogenic *E. coli* contamination in mung bean sprouts.

Endophytes are microbes living in the internal tissues of plants without causing any overt negative effects on the plants (Bacon and White, 2002). *Bacillus* spp. are some of the most frequently isolated endophytes, reportedly from apple (Bulgari et al., 2012), banana (Andrade et al., 2014; Souza et al., 2014), Chinese cabbage (Haque et al., 2016), cucurbit fruits (Glassner et al., 2015), grape (West et al., 2010), strawberry (de Melo Pereira et al., 2012), and walnut (Zhang et al., 2014). In this study, anti-human-pathogen *B. subtilis* endophytes, LCA1 and M24, were isolated from the surface-sanitized lettuce and mung bean seeds. It is very likely that anti-human-pathogen *B. subtilis* can also be isolated from other produce.

While the main interest in the beneficial endophytic *Bacillus* is on controlling plant diseases (Bacon and Hinton, 2002; Liu et al., 2009) and/or promoting plant growth (Bai et al., 2002; Rajendran et al., 2008), some endophytic *Bacillus* strains have been found to be inhibitory against human pathogens, including *Bacillus cereus*, *Clostridium perfringens*, *E. coli*, *L. monocytogenes*, *S. enterica* Typhimurium, and *Shigella flexneri* (Bacon and Hinton, 2002;

Földes et al., 2000; Islam et al., 2010; La Ragione et al., 2001; La Ragione and Woodward, 2003; Moore et al., 2013; Pinchuk et al., 2002; Sabaté and Audisio, 2013). However, there is little information on the use of anti-human-pathogen endophytic *Bacillus* strains as biocontrol agents to reduce the food safety risk of fresh produce. This study focused on the two endophytic *B. subtilis* strains, LCA1 and M24, isolated from lettuce and mung bean sprouts, respectively. The two strains demonstrated a similar, broad anti-human-pathogen spectrum (Table 1), which was comparable with the antimicrobial spectrum of *Pseudomonas fluorescens* 7-29, one of the most studied biocontrol agents for produce safety (Fett, 2006; Liao, 2008; Matos and Garland, 2005b). It is unknown if *Pseudomonas* is safe for human consumption. In contrast, *B. subtilis* bacteria are not known to be pathogenic; in fact, *B. subtilis* have been used as probiotics that are beneficial to human health (Khatri et al., 2016; Tompkins et al., 2010) and microbial safety of animal products (Joerger and Ganguly, 2017).

It is well documented that *B. subtilis* can produce more than 45 antimicrobial substances predominately, including ribosomally synthesized peptides (e.g. bacteriocins) and peptides formed non-ribosomally (Baruzzi et al., 2011; Mongkolthanaruk, 2012; Stein, 2005). As demonstrated by using the deferred agar spot method (Fett, 2006) and their *cat*-tagged derivatives, the isolates LCA1 and M24 could produce diffusible substances active against all pathogens tested, including both Gram-positive and -negative pathogens (Table 1). However, the properties of the antimicrobial substances remain to be studied. On the other hand, the laboratory strain 168 displayed on the DSM agar an inhibition only against *L. monocytogenes*, due to its anti-*Listeria* subtilisin (Zheng et al., 2000).

Production of an antimicrobial substance from *B. subtilis* can be affected by growth conditions, such as medium composition, growth temperature, and bacterial physiological state.

Moore et al. (2013) reported that seven wild *B. subtilis* strains displayed inhibitory activity against pathogens only on starch agar at 30°C, but not at 37 °C or on trypticase soy agar (TSA) or nutrient agar at either 30 °C or 37 °C. In other studies, *B. subtilis* 168 was found to produce bacilysocin on TSA immediately before or early in sporulation, respectively (Stöver and Driks, 1999; Tamehiro et al., 2002). In our preliminary tests, it was noticed that the inhibition zones of the *B. subtilis* isolates against the *S. enterica* or *E. coli* indicator on LB agar plates were generally smaller than those on DSM agar plates. In addition, on the DSM plates, *B. subtilis* 168 did not show any inhibitory activity against the tested pathogens except for *L. monocytogenes* Scott A (Table 1), but *B. subtilis* 168 was active against both *S. enterica* and EHEC cocktails in LB broth (Fig. 1) and during the mung bean seed sprouting (Fig. 4). The disparity might due to the differences in growth matrix and/or temperature (*in vitro* at 37 °C vs. *in planta* at room temperature). These data, as well as those of others, suggest that the antimicrobial activity of *B. subtilis* might differ significantly *in vitro* from *in planta* (i.e., mung bean sprouts) and might be affected greatly by growth conditions. This should be taken into account when applying *B. subtilis* as a biocontrol agent in field-growing produce, where the environment is much more complex and less controllable than the conditions of mung bean sprouting.

This study demonstrated that both *B. subtilis* and the human pathogens, *S. enterica* and EHEC, could internalize in mung bean sprouts during sprouting (Figs. 2 and 3). However, the location of the internalized bacteria is yet unclear. With immunocytochemical techniques, *E. coli* O157:H7 cells were observed primarily in the spaces between cells in every major tissue (cortex, phloem, xylem, epidermis, and pith) in the hypocotyls of mung bean sprouts (Deering et al., 2011). Using the same technique, Gu et al. (2011) demonstrated the presence of *S. enterica* Typhimurium in the phloem and other tissues of tomato plants. Using a confocal microscope,

Gandhi et al. (2001) identified *S. enterica* Stanley at the depth of 12 μm within alfalfa sprouts. It is ideal for a biocontrol agent to be able to localize in different portions throughout the plants being treated or at least to reside in the portions where the human pathogens can colonize.

This study found internalized *B. subtilis* was able to keep at a relatively stable concentration during the entire sprouting period, regardless of the presence of pathogens, which may be significant as a biocontrol agent. By contrast, the *Salmonella* or EHEC population decreased over time, even at the absence of a *B. subtilis*. A similar trend was observed in *Arabidopsis thaliana* and lettuce, and reported to be due to the presence of indigenous competitors (Cooley et al., 2003; Erickson et al., 2010a; Erickson et al., 2010b). The indigenous competitors of mung bean beans might have caused the aforementioned decreases, although the mechanism is unclear at this point. Regardless, our data illustrated that pre-inoculation of the three *B. subtilis* strains could significantly further reduce *S. enterica* or EHEC internalization in mung bean sprouts (Fig. 4). The inhibitory effects might have resulted from the single use or a combination of these mechanisms: competing for nutrients, releasing antimicrobial substances, or stimulating the mung bean sprout innate immunity (Iniguez et al., 2005). Since the relative inhibitory strength of LCA1-*cat*, M24-*cat*, and 168-*cat* seen in mung bean sprouts corresponded to the relative inhibition radii measured on the DSM agar plates (Table 1), the inhibitory effects of the three strains in the sprouts were likely due to their antimicrobial substances. However, the actual mechanism remains to be explored.

Because LCA1-*cat* was the most effective, more potent than the mung bean isolate M24-*cat*, the source (lettuce vs. mung beans) of the *Bacillus* appeared not to affect the efficacy of the *Bacillus* isolates in controlling the internalization of *S. enterica* or EHEC in mung bean sprouts. This observation suggests that LCA1 may be used in other produce to control the pathogen

internalization. Since the *Bacillus* did not completely abolish the internalized pathogens in the sprouts (although the challenge doses of the pathogens were high and are rarely encountered in real life), it may need to combine this biocontrol method with the FDA's seed decontamination method to archive the optimal control.

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Fig. 1. Effects of the *Bacillus* antagonists on the growth of *S. enterica* (A) or EHEC (B) cocktail in the LB broth

The population means (lg CFU/ml) and the standard deviation bars of *S. enterica* or EHEC are results of three independent experiments. The same letter is indicative of no significant difference ($P > 0.05$).

Fig. 2. Internalization of the *Bacillus* antagonists, and *S. enterica* or EHEC cocktail in mung bean sprouts

The population means (lg CFU/g of seeds) and the standard deviation bars of the internalized bacteria are results of three independent experiments. The same letter is indicative of no significant difference ($P > 0.05$).

Fig. 3. Effects of *S. enterica* and EHEC on internalization of the *B. subtilis* antagonists in mung bean sprouts

The population means (lg CFU/g of seeds) and the standard deviation bars of the internalized *B. subtilis* LCA1-cat (A), M24-cat (B), and 168-cat (C) are results of three independent experiments. No significant effect of either pathogen ($P > 0.05$) was observed on the internalization of any of the *B. subtilis* antagonists at all sampling time.

Fig. 4. Inhibition of internalization of the *S. enterica* (A) or EHEC (B) cocktail by the internalized *B. subtilis* antagonists in mung bean sprouts

The population means (lg CFU/g of seeds) and the standard deviation bars of the internalized *S. enterica* or EHEC are results of three independent experiments. The same letter is indicative of no significant difference ($P > 0.05$).

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 3. (continued)

Fig. 4.

Table 1. Antimicrobial spectra of LCA1-cat, M24-cat, and 168-cat

Pathogen strain	Radius of inhibition zone (mm)* $\pm$ SD**		
	LCA1-cat	M24-cat	168-cat
<i>S. enterica</i> Choleraesuis	8.3 $\pm$ 1.3 (C)	4.8 $\pm$ 0.4 (T)	0
<i>S. enterica</i> Senftenberg O&B	9.4 $\pm$ 1.0 (C)	6.5 $\pm$ 1.1 (T)	0
<i>S. enterica</i> Thompson	9.1 $\pm$ 1.2 (C)	5.2 $\pm$ 0.4 (C)	0
<i>S. enterica</i> Berta	5.8 $\pm$ 1.1 (C)	3.7 $\pm$ 0.5 (C)	0
<i>S. enterica</i> Rubislaw	6.0 $\pm$ 1.2 (T)	5.8 $\pm$ 0.4 (T)	0
<i>S. enterica</i> Typhimurium LT2	8.0 $\pm$ 0.7 (C)	4.2 $\pm$ 0.9 (T)	0
<i>S. enterica</i> Typhimurium ATCC53648	5.9 $\pm$ 2.5 (T)	3.5 $\pm$ 0.8 (C)	0
<i>S. enterica</i> Typhimurium ATCC14028	12.0 $\pm$ 1.5 (C)	8.7 $\pm$ 0.9 (C)	0
<i>S. enterica</i> Typhimurium ATCC13311	4.1 $\pm$ 0.7 (T/C)**	3.8 $\pm$ 0.4 (T)	0
<i>S. enterica</i> Typhimurium ATCC13036	5.4 $\pm$ 1.2 (C)	2.8 $\pm$ 0.4 (C)	0
<i>E. coli</i> O104:H TW04909	7.8 $\pm$ 1.7 (C)	5.3 $\pm$ 0.5 (C)	0
<i>E. coli</i> O104:H ECOR 228	8.0 $\pm$ 2.0 (C)	4.8 $\pm$ 0.7 (C)	0
<i>E. coli</i> O 145:H NT D177	5.1 $\pm$ 0.4 (C)	2.7 $\pm$ 0.5 (T)	0
<i>E. coli</i> O 121:H19 3377-85	4.8 $\pm$ 1.1 (T)	4.5 $\pm$ 0.8 (T)	0
<i>E. coli</i> O 111:H2 RD8	5.4 $\pm$ 2.1 (T)	2.0 $\pm$ 0.6 (T)	0
<i>E. coli</i> O 103:H2 MT#80	10.1 $\pm$ 1.6 (C)	7.7 $\pm$ 1.5 (C)	0
<i>E. coli</i> O 45:H2 M103-19	11.0 $\pm$ 1.0 (C)	9.7 $\pm$ 2.3 (T/C)**	0
<i>E. coli</i> O 26:H11 DEC10B	12.8 $\pm$ 3.8 (T)	7.8 $\pm$ 1.7 (C)	0
<i>E. coli</i> O157:H7 G5310	6.9 $\pm$ 1.2 (C)	5.5 $\pm$ 0.5 (T)	0
<i>E. coli</i> O157:H7 505B	3.8 $\pm$ 0.5 (C)	3.0 $\pm$ 0.6 (C)	0
<i>L. monocytogenes</i> Scott A	14.8 $\pm$ 3.1 (C)	14 $\pm$ 3.9 (C)	4 $\pm$ 1.0 (C)
<i>S. aureus</i>	5.7 $\pm$ 1.1 (C)	8 $\pm$ 0.8 (T)	0
<i>K. pneumoniae</i> 700603	4.0 $\pm$ 0.8 (C)	3 $\pm$ 1.2 (T)	0

*C: clear inhibitory zone; T: turbid inhibitory zone; results are the means of triplicate tests.

**SD: standard deviation

*** Turbid in two experiments but clear in the third.