



# Lactic Acid Bacteria from Fermented Asparagus and Stinky Beans Inhibit Clinical Diarrheagenic *Escherichia coli* and Clinical Methicillin-Resistant *Staphylococcus aureus*

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**Abstract:** The probiotics exhibiting antagonistic activity against gastrointestinal pathogenic bacteria are essential for protecting the host from illnesses and regulating intestinal balance. In this study, we successfully isolated 7 lactic acid bacteria (LAB) from fermented asparagus and fermented stinky beans. They showed the sign of probiotic properties, especially 2 strains from fermented asparagus, PZ12 and PZ14, strongly tolerated to simulated gastric juice pH 3.0 supplemented with 0.3% pepsin. Additionally, these 2 LAB strains tolerated 0.5% bile salts for up to 3 hours. Antagonistic activity of 7 LAB strains against clinical Diarrheagenic *Escherichia coli* (DEC) and clinical MRSA in this study showed that all LAB strains were capable of inhibiting clinical DEC and clinical MRSA by providing an inhibition zone in the range between 22 and 39 mm. PZ12 and PZ14 also displayed relatively wide inhibition zones against these intestinal pathogens. Antimicrobial-resistant examination demonstrated that most LAB strains could be destroyed by most of the antimicrobial agents tested. LAB strains PZ12 and PZ14 were shown to be resistant to three antimicrobial agents. PZ12 could resist ciprofloxacin, fosfomycin, and streptomycin, and PZ14 was resistant to ciprofloxacin, ceftiofur, and streptomycin. Hence, pickles are a good source of beneficial probiotics for humans.

**Keywords:** Lactic acid bacteria (LAB); fermented asparagus; fermented stinky beans; Diarrheagenic *Escherichia coli* (DEC); clinical MRSA

## 1. Introduction

Diarrheagenic *Escherichia coli* (DEC) is an important bacterial group that plays a role in gastrointestinal tract infections, resulting in morbidities and mortalities worldwide [21]. DEC consists of 6 pathotypes, *e.g.*, enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), including the most important pathotype, enterohemorrhagic *E. coli* (EHEC). EHEC is defined by the presence of the coding for intimin, the protein involved with bacterial attachment, and *stx* genes encode toxins called Shiga toxins (Stx1 and Stx2), causing bloody diarrhea and kidney failure, leading to death in complicated cases. Therefore, amongst 6 DEC pathotypes, EHEC shows the most devastating impact on humans. Shiga toxin-producing *E. coli* (STEC), defined by the presence of the sole *stx* genes (*stx1* or *stx2* or both), can cause symptoms

similar to those of EHEC. In addition, another group of bacteria that can cause food poisoning is *Staphylococcus aureus*, containing staphylococcal enterotoxins (SEs), including toxic shock syndrome toxin (TSST-1), which is a member of the pyrogenic toxin superantigen (PTSAg) family [13]. Staphylococcal superantigens can stimulate the massive release of cytokines from T-lymphocytes and macrophages [20], leading to the excess of cellular immune responses causing toxic shock [33]. In addition, they can cause staphylococcal pneumonia and staphylococcal purpura fulminans [8, 14]. More importantly, methicillin-resistant *Staphylococcus aureus* (MRSA), the *S. aureus* strain carrying *mecA*, has emerged and spread worldwide [2, 4, 36]. It shows resistance to numerous antimicrobial drugs, resulting in trouble with therapeutic approaches.

Lactic acid bacteria (LAB) with probiotic potentials are considered promising solutions to regulate the balance of gut microbiota, leading to the proper work of the gastrointestinal tract. Also, they demonstrate the health-promoting effects on the hosts, for instance, lowering cholesterol and producing  $\gamma$ -aminobutyric acid (GABA), which plays a role as a diabetic suppressant and anti-hypertension [16]. Since LAB is generally recognized as safe (GRAS), LAB is thus widely consumed to promote health and prevent gastrointestinal tract infections. The pickles are the source of excellent probiotic strains that benefit human health. Therefore, this study aims to search for probiotic bacteria from pickled asparagus and stinky beans, which are commonly and widely consumed in southern Thailand. Their antagonistic capability is explored for the benefit of public health.

## 2. Materials and Methods

### 2.1 Indicator bacteria

Indicator bacteria used for antagonistic examination were 4 DEC strains (3 from Hat-Yai Hospital and 1 from beef) and 1 clinical MRSA strain from a patient in Songklanagarind Hospital. Characteristics of these indicator bacteria are listed in Table 1. The Ethics Committee of the Faculty of Medicine, Prince of Songkla University, Thailand (EC no. 56-225-19-2-3) approved the research protocol to collect these bacteria.

### 2.2 LAB isolation

To isolate LAB, 10 pickle samples (stinky beans and asparagus) were collected from fresh markets in Hat-Yai city, and all of them were processed within 2 hours as described previously [16] with modifications. Briefly, 10 g of pickle was mixed with 90 mL of 0.85% (w/v) sodium chloride solution (normal saline solution, NSS), and 0.1 mL of the solution was spread on Lactobacilli MRS agar (Difco, USA) fortified with 400 mg/L of bromocresol purple (BCP) and incubated at 30°C for 48 hours under microaerophilic condition. Typical yellowish Lactobacilli colonies were randomly collected to test the absence of catalase. They were also subjected to examine the characteristics of Gram-positive rod-shaped cells. For further analyses, LAB strains were stored in 20% (v/v) glycerol at -80 °C.

### 2.3 Investigating the probiotic properties of LAB strains

#### 2.3.1. Toleration of LAB to simulated gastric juice

The gastric system was simulated to examine the toleration of LAB strains in gastric juice. Phosphate buffer saline (PBS), pH 2.0 and pH 3.0, supplemented with 0.3% (w/v) pepsin (Sigma-Aldrich, USA), were prepared. The experiment was performed as described by Wang et al. [34] with slight modifications. Briefly, 1 mL of  $1.5 \times 10^9$  CFU/mL bacterial culture was added into 9 mL of 0.3% (w/v) pepsin-supplemented PBS (pH 2.0 and pH 3.0) and incubated at 37°C. The bacterial count was carried out at 0, 90, and 180 minutes by surface plate count on Lactobacilli MRS agar as described above. A 0.3% (w/v) pepsin-supplemented PBS, pH 6.2, was used as a control. The experiment was performed in triplicate.

#### 2.3.2. Toleration of LAB to bile salt

Bile salt was used to simulate the condition of the human intestinal tract. LAB strains were tested for toleration in 2 bile salt concentrations, 0.3% and 0.5%. The experiment was performed as previously described by Tulini et al. [31] with slight modifications. In short, a working bacterial culture of  $1.5 \times 10^9$  CFU/mL was prepared as described above. One milliliter of working culture was spiked into 99 mL of sterile PBS supplemented with 0.3% and 0.5% (w/v) of bile salt (Sigma-Aldrich, USA) and incubated statically at 37°C for 3-time points, 0, 90, and 180 minutes. As described above, bacterial survival was assessed by surface plate

count on Lactobacilli MRS agar. A sterile PBS without bile salt was used as a control. The experiment was performed in triplicate.

#### 2.4 Inhibition of DEC and MRSA by LAB strains

The antagonistic activity of the LAB strains was measured using agar spot assay as previously described by Armas et al. [1]. Briefly, overnight culture of the pathogens (DEC and MRSA) was diluted in Brain Heart Infusion (BHI) broth (Difco, USA). A 0.1 mL of each pathogen culture (approximately  $1.5 \times 10^6$  CFU/mL) was spread onto BHI plates. The plates were left to dry for 15 minutes at room temperature. Overnight cultures of the LAB strains grown in Lactobacilli MRS broth for 48 hours were adjusted to  $1.5 \times 10^5$  CFU/mL, and a 3  $\mu$ L of the diluted culture was spotted on the agar surface containing pathogen inoculated. The experiment was performed in triplicate. Plates were left for 5 minutes for drying at ambient temperature and then incubated aerobically at 37°C for 24 hours. Vernier caliper measured the inhibition zone.

#### 2.5 Determination of antimicrobial susceptibility of LAB strains

With slight modifications, an antimicrobial susceptibility test was performed using the disk diffusion method, as described by Duskova et al. [7]. Bacterial suspensions with turbidity equivalent to 0.5 McFarland standards were swabbed evenly onto Lactobacilli MRS agar plates. Twelve common antibiotic disks, amikacin (30  $\mu$ g), chloramphenicol (30  $\mu$ g), ciprofloxacin (5  $\mu$ g), clindamycin (2  $\mu$ g), gentamicin (10  $\mu$ g), erythromycin (30  $\mu$ g), fosfomycin (200  $\mu$ g), ceftiofur (30  $\mu$ g), imipenem (10  $\mu$ g), streptomycin (10  $\mu$ g), cotrimoxazole (23.75/1.25  $\mu$ g), and tetracycline (30  $\mu$ g) (Oxoid, Basingstoke, UK), were placed on Lactobacilli MRS agar plates. The plates were incubated at 30°C for 24 hours under microaerophilic conditions. Inhibition zone diameters, including the diameter of the disk, were measured.

#### 2.6 Statistical analysis

Multivariate analysis of variance (ANOVA) was used to analyze the toleration of LAB to simulated gastric juice and the toleration of LAB to bile salt. A significant difference was set at  $p$ -value < 0.05.

**Table 1.** Characteristics of indicator bacteria (pathogens) used in this study

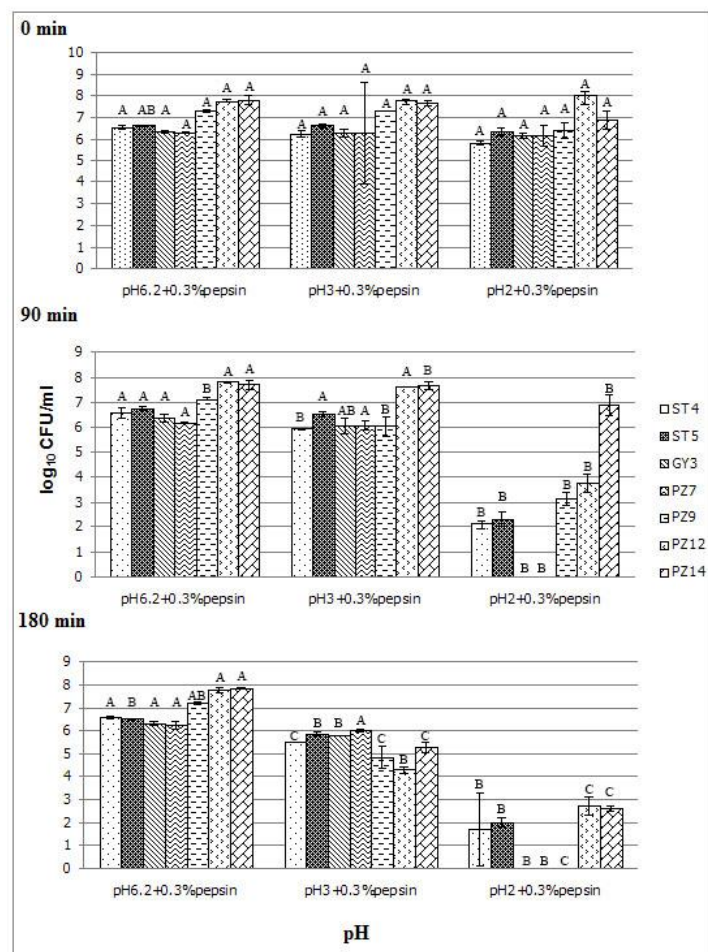
| Bacterial strain | DEC Pathotype | Serotype | Origin (year)   | Virulence trait   | Reference |
|------------------|---------------|----------|-----------------|---|-----------|
| PSU1             | STEC          | O8       | Beef (2012)     | <i>stx1</i> <sup>+</sup> [ <sup>a</sup> RPLA titer = 128], <i>stx2</i> <sup>+</sup> [RPLA titer = 16]                             | [29]      |
| EDL933           | EHEC          | O157     | Human (1982)    | <i>stx1</i> <sup>+</sup> [RPLA titer = NA], <i>stx2</i> <sup>+</sup> [RPLA titer = 2,048], <i>eae</i> <sup>+</sup>                | [25]      |
| PE-27            | EPEC          | O111     | <sup>b</sup> NA | <i>bfp</i> <sup>+</sup> , <i>eae</i> <sup>+</sup>   | [24]      |
| PSU192           | ETEC          | O169     | Human (2014)    | <i>est</i> <sup>+</sup> , <i>astA</i> <sup>+</sup>  | [27]      |
| PSU263           | EAEC          | O127a    | Human (2014)    | <i>aggR</i> <sup>+</sup> , <i>aggA</i> <sup>+</sup> , <i>astA</i> <sup>+</sup>  | [28]      |
| MRSA PSU20       | NA            | NA       | Human (2014)    | <i>mecA</i> <sup>+</sup> , <i>spa</i> <sup>+</sup> , <i>femB</i> <sup>+</sup> , <i>sea</i> <sup>+</sup> , <i>coa</i> <sup>+</sup> | [30]      |

<sup>a</sup>RPLA, reverse passive latex agglutination test to quantify the amount of Stx production. <sup>b</sup>NA, Not applicable.

### 3. Results and Discussion

#### 3.1 LAB isolation and their toleration to simulated gastric juice

In the course of LAB investigation from pickle samples, 2 LAB strains (ST4 and ST5) were successfully obtained from fermented stinky beans and 5 (GY3, PZ7, PZ9, PZ12, PZ14) from fermented asparagus. They were Gram-positive rod-shaped bacteria, presenting the typical characteristic of LAB on Lactobacilli MRS agar. Focusing on gastric juice toleration of LAB, some bacterial strains resisted simulated gastric juice. They could withstand 0.3% pepsin at pH 3 for up to 180 minutes without much reduction of bacterial population. Although at pH 2, LAB strains GY3 and PZ7 could not survive at 90 minutes, strains ST4, ST5, PZ9, and PZ12 could still survive until 180 minutes (Figure 1). Particularly, the bacterial population of PZ14 remained at the approximate amount ( $10^7$  log CFU/mL) at 90 minutes compared to the starting time (0 minutes). At 180 minutes, LAB strain PZ14 survived at approximately  $10^3$  log CFU/mL (Figure 1). This demonstrates the ability of the LAB to withstand stomach acidity, suggesting their strength to survive gastric transit.



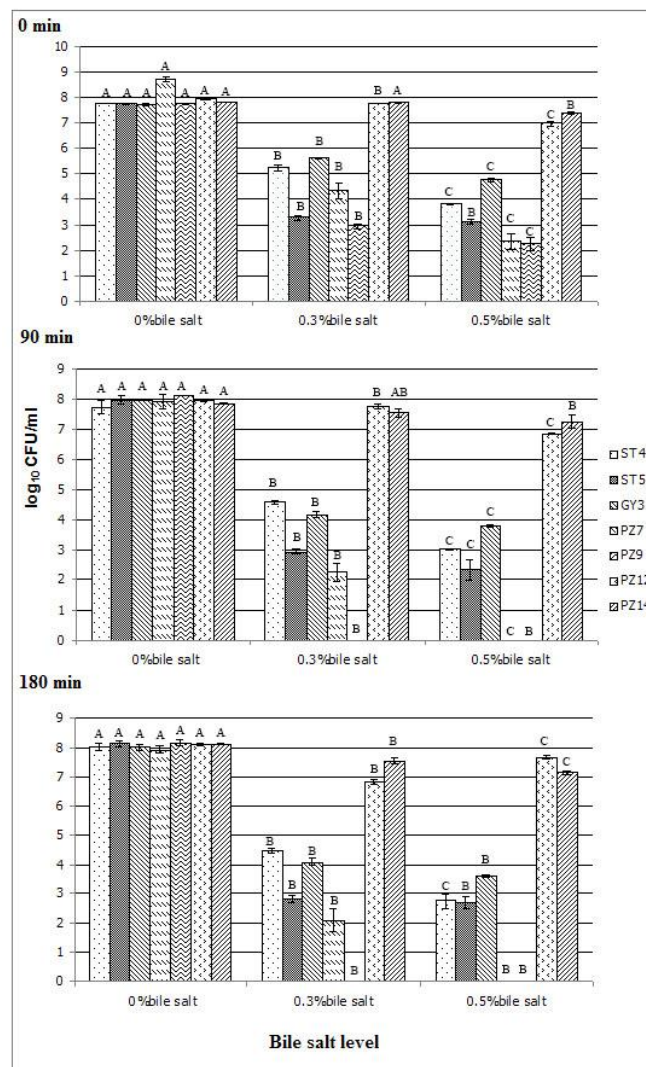
**Figure 1.** Toleration of LAB strains isolated from fermented asparagus and fermented stinky beans to simulated gastric juice supplemented with 0.3% pepsin, pH 3.0, and pH 2.0 for 90 minutes and 180 minutes. Upper case letters indicate the significant difference in simulated gastric juice toleration of LAB strains among three-time points: 0 min (control), 90 min, and 180 min ( $p$ -value < 0.05).

There are several mechanisms that LAB uses for acid toleration. For example, (i) acid neutralization process by arginine dihydrolase system (ADS) by which the LAB produces alkaline substances such as urea, arginine, and ammonia to nullify acid, (ii) production of biofilm to protect the cells, and (iii) proton pump by

F1-F0- ATPase that hydrolyzes or synthesizes the ATP by F1 protein and transport proton through F0 complex [35]. In addition, stress response, such as the production of cold shock proteins (Csps) that act as RNA chaperones to prevent RNA secondary structure and promote its biological roles, may protect LAB from the destruction by acid [15].

### 3.2 Toleration of LAB to bile salt

Bile salt is a bio-compound secreted from the liver to help digest lipids. It also has antibacterial activity that leads to stress on bacteria through multiple mechanisms, for instance, disruption of bacterial cell membranes, denaturation of proteins, chelating the iron and calcium, and causing oxidative damage to DNA [32]. Therefore, LAB with probiotic potentials must endure the antibacterial activity of bile salts to establish itself in the human gut. Focusing on the resistance to bile salt at 0.3% and 0.5%, it was found that strains ST4, ST5, GY3, PZ7, and PZ9 had a reduction in numbers since 0 minutes, which were contrasted to the strains PZ12 and PZ14, exhibiting almost no reduction at 0 minutes. More importantly, these two strains showed a slight decrease in bacterial population at 90 minutes and 180 minutes for both bile salt concentrations (Figure 2). This result suggests LAB's ability to establish itself in the human intestine.



**Figure 2.** Toleration of LAB strains isolated from fermented asparagus and fermented stinky beans to bile salts (0.3% and 0.5%) for 90 minutes and 180 minutes. Uppercase letters indicate the significant difference in bile salt toleration of LAB strains among three bile salt concentrations: 0 % (control), 0.3%, and 0.5% ( $p$ -value < 0.05).



Bile salt is a biological compound synthesized in the liver from cholesterol. It shows a strong antimicrobial activity by destroying bacterial cell membranes and triggering DNA damage. LAB with probiotic potentials must be able to resist these mechanisms to inhabit the host's intestine [26]. The bacterial response to bile salt is a multi-factorial event. Active efflux of bile salts [23], bile salts hydrolysis [18], and the changes in cell membrane/cell wall compositions [9] are the most common bile tolerance mechanisms in *Lactobacillus* and *Bifidobacterium* [26]. Our results aligned with the Hermanns et al. [11] study. They isolated six LAB strains from artisan cheeses in Brazil and tested their toleration to 0.3% and 1.0% bile for 4 hours. It was found that there was a 1 to 2-log cycle reduction in bacterial survival compared to the control group (no bile). Hayisama-ae et al. [10] also demonstrated approximately 2 log cycle reductions in *Lactobacillus plantarum* strain DW12 survival after exposure to 0.3% bile salts for 6 hours. The LAB reduction of about 1 to 2 log cycles after LAB exposure to bile salts is thought to be common. A maximum of 1.5 log reduction from the initial count is a criterion defined by Charteris et al. [3]. Therefore, our LAB strains in this study demonstrate a strong toleration to bile salts and are believed to inhabit the intestine.

### 3.3 Inhibition of DEC and MRSA by LAB

The inhibition of pathogens is thought to be an essential weapon for the consumption of probiotics. The results of the antagonistic activity of 7 LAB strains against DEC and clinical MRSA in this study showed that all LAB strains could inhibit DEC and clinical MRSA by providing an inhibition zone between 22 and 39 mm (Table 2), focusing on LAB strains PZ12 and PZ14, which exhibited high probiotic potentials due to their ability to tolerate 0.3% pepsin and bile salt for extended time. It was found that both strains demonstrated relatively wide inhibition zones (wider than 30 mm) in all strains of DEC tested. As for the MRSA PSU20, all LAB demonstrated similar zone diameters ranging from 22 to 27 mm (Table 2). The distinct antagonistic activity of LAB against DEC and MRSA in this present study indicates that LAB produces a wide variety of compounds, e.g., organic acids, hydrogen peroxide, and bacteriocins. These compounds are documented to be capable of inhibiting spoilage and pathogens, such as Gram-positive or Gram-negative bacteria [6]. Hayisama-ae et al. [10] also investigated the antibacterial activity of *Lactobacillus plantarum* DW12 isolated from red seaweed against numerous pathogens and found that DW12 could inhibit all pathogens tested. A similar result was observed in the work of Makras et al. [19], who demonstrated the antibacterial activity of *Lactobacilli* against *Salmonella enterica* serovar Typhimurium through the production of organic acid, mainly lactic acid.

**Table 2.** Antagonistic activity of LAB strains isolated from pickles on diarrheagenic *E. coli* and clinical methicillin-resistant *S. aureus* by agar spot assay

| LAB strain | Inhibition zone diameter (mm) |             |            |             |             |            |
|------------|-------------------------------|-------------|------------|-------------|-------------|------------|
|            | STEC PSU1                     | EHEC EDL933 | EPEC PE-27 | ETEC PSU192 | EAEC PSU263 | MRSA PSU20 |
| ST4        | 31.80                         | 30.70       | 27.80      | 26.70       | 25.60       | 23.30      |
| ST5        | 30.00                         | 28.40       | 34.50      | 26.90       | 32.50       | 24.10      |
| GY3        | 35.00                         | 27.10       | 30.40      | 25.00       | 29.00       | 21.50      |
| PZ7        | 37.00                         | 33.90       | 39.00      | 33.80       | 30.00       | 24.60      |
| PZ9        | 35.20                         | 31.70       | 36.40      | 33.60       | 29.60       | 21.50      |
| PZ12       | 32.50                         | 32.30       | 39.00      | 32.80       | 32.90       | 26.50      |
| PZ14       | 36.00                         | 34.40       | 34.90      | 34.70       | 34.90       | 23.00      |

### 3.4 Determination of antimicrobial susceptibility of LAB

Antimicrobial susceptibility assay of LAB demonstrated that all LAB strains provided an inhibition zone for most of the antimicrobial drugs tested, e.g., amikacin, chloramphenicol, gentamicin, clindamycin, erythromycin, imipenem, cotrimoxazole, and tetracycline. This suggested that these antimicrobial agents tested can destroy most LAB strains. Nevertheless, there were four drugs that most of the LAB showed resistance pattern: ciprofloxacin, fosfomycin, ceftioxin, and streptomycin (0 mm of inhibition zone) (Table 3). LAB strains PZ12 and PZ14 were resistant to three antimicrobial agents. PZ12 could withstand ciprofloxacin, fosfomycin, and streptomycin, and PZ14 was resistant to ciprofloxacin, ceftioxin, and streptomycin (Table 3).

Duskova et al. [7] examined antimicrobial resistance in lactobacilli isolated from Czech Republic foods using disk diffusion and broth microdilution methods. They found 15 strains (17%) resistant to at least one antimicrobial agent and one multi-drug resistant strain. In addition, among resistant strains, they were resistant to gentamicin at the highest frequency, 7.8%. Gentamicin resistance in these bacterial strains concurs with a study by Danielsen and Wind [5] and Nawaz et al. [22] that also found high gentamicin resistance. Our present study showed that most of the LAB isolated from pickles were resistant to ciprofloxacin, fosfomycin, ceftiofur, and streptomycin. These results are concordant with the work of Karapetkov et al. [17], who investigated the drug-resistant pattern of four *Lactobacillus* spp. and one *Streptococcus thermophilus* from dairy products and fruits and found that they were suppressed by chloramphenicol, erythromycin, and tetracycline.

**Table 3.** Antimicrobial susceptibility of LAB strains from pickled asparagus and stinky beans

|      | Clear zone (mm) |       |                |       |       |       |      |       |       |      |       |       |
|------|-----------------|-------|----------------|-------|-------|-------|------|-------|-------|------|-------|-------|
|      | <sup>a</sup> AK | C     | CIP            | CN    | DA    | E     | FOS  | FOX   | IPM   | S    | SXT   | TE    |
| ST4  | 18.00           | 35.10 | <sup>b</sup> 0 | 17.10 | 17.15 | 37.00 | 0    | 0     | 51.00 | 0    | 25.40 | 29.50 |
| ST5  | 17.20           | 35.60 | 0              | 19.00 | 12.00 | 38.10 | 0    | 0     | 40.50 | 8.00 | 25.40 | 31.70 |
| GY3  | 10.45           | 34.00 | 0              | 14.50 | 23.30 | 34.50 | 0    | 0     | 47.20 | 0    | 21.00 | 29.40 |
| PZ7  | 11.10           | 34.00 | 11.10          | 13.60 | 17.00 | 35.70 | 0    | 0     | 46.60 | 0    | 20.25 | 31.00 |
| PZ9  | 19.05           | 34.30 | 10.20          | 16.80 | 9.00  | 38.00 | 0    | 0     | 48.00 | 9.45 | 29.20 | 29.00 |
| PZ12 | 9.30            | 32.10 | 0              | 11.00 | 14.00 | 33.70 | 0    | 10.00 | 47.80 | 0    | 23.25 | 26.00 |
| PZ14 | 8.15            | 32.00 | 0              | 11.80 | 14.00 | 33.00 | 8.30 | 0     | 47.00 | 0    | 22.20 | 25.50 |

<sup>a</sup>AK, amikacin; C, chloramphenicol; CIP, ciprofloxacin; CN, gentamicin; DA, clindamycin; E, erythromycin; FOS, fosfomycin; FOX, ceftiofur; IPM, imipenem; S, streptomycin; SXT, cotrimoxazole; TE, tetracycline. <sup>b</sup>0, no clear zone.

The presence of antimicrobial-resistant phenotypes in LAB in this current study seems unsafe for consumers. However, the LAB isolated from probiotic products is usually reported to resist numerous antimicrobials [10], and the antimicrobial resistance in probiotics may be able to provide benefits to the host who has intestinal imbalance due to the antimicrobials used [12].

## 4. Conclusions

This study isolated LAB strains with good probiotic potentials from fermented asparagus and stinky beans. They exhibited antagonistic activity against clinical DEC and clinical MRSA with the capability of causing food poisoning, suggesting that they were equipped with pivotal weapons that can protect us, at least in part, from gastrointestinal pathogens. Antimicrobial resistance in LAB is common and thought to be beneficial to the host with intestinal imbalance during drug use. Therefore, pickled is an abundant source of probiotics. This study encourages the search for LAB strains from pickles with stronger probiotic properties that are useful for humans.

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**Author Contributions:** PR, original draft preparation, review, editing; WK, methodology, technical assistance; WP, technical assistance; PS, conceptualization, original draft preparation, editing, validation, resource. All authors have read and agreed to the published version of the manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

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