Probiotic characterization of *Bacillus subtilis* SM10.1

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**Abstract.** As compared to common probiotics such as lactic acid bacteria, Bacillus species has the advantage of being able to produce spores that withstand extreme conditions and long storage period. One of the Bacillus species that has been reported to have probiotic properties is *Bacillus subtilis*. However, different bacterial strains of the same species might exhibit completely different abilities. Therefore, the specific properties of individual strains should be well-defined. In this study, *Bacillus subtilis* SM10.1 were examined *in vitro* for its probiotic properties such as low pH tolerance, bile salt resistance, antibiotic susceptibility, antimicrobial activity, auto- and co-aggregation, and adhesion ability to epithelial cells. Based on the results, spores of *B. subtilis* SM10.1 showed more tolerance to low pH than vegetative cells. Nevertheless, the vegetative cells of *B. subtilis* SM10.1 were resistant to bile salts. *B. subtilis* SM10.1 exhibited antimicrobial activity and high auto-aggregation ability. Its co-aggregation ability with *Escherichia coli* and *Staphylococcus aureus* was also strong. *B. subtilis* SM10.1 demonstrated adherence ability to the buccal epithelial cell and was only resistance to streptomycin, bacitracin, and clindamycin among twenty-three kinds of antibiotics. The results of this study suggested that *B. subtilis* SM10.1 has potential as a probiotic strain. However, further investigation is required for the use of *B. subtilis* SM10.1 in human.

1. **Introduction**

According to FAO/WHO (2001), probiotics are defined as 'live microorganisms that can give health benefit to its host in adequate amount' [1]. Consequently, probiotic-containing products are widely used for human nutrition and a feed supplement in farms and aquaculture. The health benefits claimed for humans include a reduced incidence of diarrhea, lactose intolerance, and allergic reaction [2]. Meanwhile, probiotics as an animal feed supplement are claimed to increase feed digestibility, maintain the balance of animal gut microbiota, increase animal growth and the quality of animal products [3].

*Bacillus* species offer some advantages over the common probiotics, such as *Lactobacillus* and *Bifidobacterium*, in that they could produce spores that have the capacity to resist extreme conditions and long periods of storage. In addition, *Bacillus* could excrete some beneficial substances, e.g., degrading enzymes such as amylase and protease, antimicrobial compounds, and vitamins [4]. *Bacillus* species that have been used commercially as probiotics for human consumption, namely *B. clausii* (Enterogermina®, Sanofi), *B. subtilis*, and *B. licheniformis* (Biosporin, Biopharma). Although *Bacillus* species have been proven to promote the host's health, the effect of probiotics is strain specific. Bacteria from the same genus and/or species does not necessarily produce the same probiotic effects [5].

*Bacillus* sp. are among the widespread microorganism in nature since they can be found in soil, water, and air. Apart from those sources, *Bacillus* species are also identified as one of the bacteria in the honeybee gut microbiome [6]. The honeybee is an herbivorous insect that produces honey from plant...
nectar and pollen. The gut microbiome of honeybees has been associated with honey and bee bread production, which acts as a food source for their colony [7]. In this study, *B. subtilis* SM10.1 isolated from honeybee was characterized for its potential as probiotic.

2. Methods

2.1. Acid and bile tolerance assessment
Tolerance to low pH and bile salts was analyzed according to the method modified from Jeon *et al.* [8]. *B. subtilis* SM10.1 was grown overnight in Nutrient Broth (NB; Merck, Germany). For acid tolerance assessment, the culture was inoculated into NB with an adjusted pH of 2, 3, and 4. For bile resistant assessment, the culture was inoculated into NB supplemented with 0.3% and 0.5% of bile salt (Oxoid, UK). All tests were performed at 37 °C, and sampling was done every hour to three hours period. Enumeration of viable cells was done by the spread plate method on Nutrient Agar (NA; Merck, Germany) after incubation at 37 °C for 18 hours.

The resistance of *B. subtilis* SM10.1 spore to acid was also analyzed. The spore collection method was adapted from Guo *et al.* [9]. *B. subtilis* SM10.1 was cultured in NB at 37 °C for 36 hours with the agitation of 120 rpm. To kill vegetative cells, the culture was heated at 80 °C for 15 minutes. The culture was centrifuged at 2,500 × g for 10 minutes, and spores were collected as a pellet. The spores were washed twice with phosphate-buffered saline (PBS; pH 7.4) and re-suspended in PBS. An aliquot of the spore solution was inoculated into NB adjusted to pH 2, the lowest pH used in this study, and incubated at 37°C. Sampling and enumeration were done as stated for the acid tolerance test.

2.2. Antimicrobial activity
Antimicrobial activity was evaluated with the cross-streaking method. *B. subtilis* SM10.1 was inoculated as a straight line on Nutrient Agar and incubated at 37 °C for 24 hours. Two pathogens, *E. coli* dan *S. aureus*, were then inoculated perpendicularly close to *B. subtilis* SM10.1. Observation of the inhibition zone was done after incubation at 37 °C for 24 hours.

2.3. Antibiotic susceptibility
Antibiotic susceptibility of *B. subtilis* SM10.1 was examined according to Clinical Laboratory Standards Institute (CLSI) guideline using the disc diffusion method described by Gu *et al.* [10]. *B. subtilis* SM10.1 was cultured in Nutrient Broth at 37°C overnight. The turbidity was adjusted to 0.5 McFarland standard in 0.85% sterile saline solution. The suspension was swabbed onto Mueller Hinton Agar (Pronadisa, Condalab, Spain) using a sterile cotton swab. *B. subtilis* SM10.1 was tested against 23 antibiotics, including protein synthesis inhibitor (tiamulin, erythromycin, tylosin, kanamycin, neomycin, streptomycin, gentamicin, chloramphenicol, lincomycin, clindamycin, and tetracycline), cell wall synthesis inhibitor (amoxicillin, ampicillin, methicillin, vancomycin, oxacillin, cefoxitin), folic acid synthesis inhibitor (sulphonamide), RNA polymerase inhibitor (rifampicin), DNA gyrase inhibitor (nalidixic acid, ofloxacin, ciprofloxacin), and cell wall and protein synthesis inhibitor (bacitracin). Antibiotics in the form of discs (Liofilchem, Italy) were placed on the surface the agar plates and were incubated at 37 °C for 18 hours.

2.4. Auto-aggregation and co-aggregation
Auto-aggregation and co-aggregation assays were adapted from Jeon *et al.* [8]. *B. subtilis* SM10.1 and two pathogens, *E. coli* and *S. aureus*, were grown in Nutrient Broth to exponential phase. The cultures were centrifuged at 2,500 × g for 10 minutes. The cell pellet was washed twice with PBS and re-suspended in PBS to adjust absorbance of 0.3 ± 0.05 at 600 nm. For an auto-aggregation test, each culture was incubated at 37°C for 4 hours. The turbidity of each culture was measured at 600 nm after one and four hours of incubation. The Auto-aggregation percentage was calculated as follows.

\[
\text{Auto-aggregation} \% = \left(1 - \frac{A_t}{A_0}\right) \times 100
\]

\(A_0\) represents sample absorbance at time zero, \(A_t\) represents sample absorbance after incubation.
For the co-aggregation test, the liquid culture of *B. subtilis* SM10.1 and pathogen were mixed (1:1) and incubated at 37 °C for 4 hours. The turbidity of mixed culture was measured at 600 nm after one and four hours of incubation. The Co-aggregation percentage was calculated as follows.

\[
\text{Co-aggregation (\%) = } \left( \frac{A_p + A_B - A_M}{2} \right) \times 100
\]

A\(_p\) and A\(_B\) respectively represent the absorbance value of the liquid culture of pathogen and *B. subtilis* SM10.1 at time zero. A\(_M\) represents the absorbance value of mixed culture after incubation time.

2.5. Adhesion to epithelial cells

The ability of *B. subtilis* SM10.1 to adhere to epithelial cells was observed by the modified method from Haukioja et al. [11]. *B. subtilis* SM10.1 was cultured in Nutrient Broth to exponential phase. The culture was centrifuged at 2,500 × g for 10 minutes. The cell pellet was washed twice with PBS and resuspended in PBS to adjust absorbance of 0.3 ± 0.05 at 600 nm. Human buccal epithelial cheek was obtained from a healthy volunteer. Epithelial cells were washed in PBS 3-5 times and suspended in PBS to 106 cell/ml concentration. The bacterial and epithelial cell suspension was mixed (1:1) and incubated at 37 °C for one hour. After incubation, the mixed suspension was centrifuged at 800 rpm for two minutes. The pellet was re-suspended in PBS. Observation of bacterial cell adhesion to epithelial cells was done under a microscope after Gram staining was applied.

3. Results and Discussion

3.1. Acid and Bile Tolerance Assessment

To exert its health benefits, probiotics have to resist extreme acidic gastric conditions and withstand the effect of bile salts secreted by the small intestine. Bile salts with detergent properties are able to dissolve fats, which are related to its antibacterial activity. Bile salts concentration in the intestine are varied between 0.2 - 2.0%, but the concentration of 0.3% is commonly used for bile salt tolerance assessment [12]. Table 1 and Table 2 show the viability of *B. subtilis* SM10.1 after exposure with acid and bile salt.

Based on Table 1, the number of *B. subtilis* SM10.1 cell experienced a significant decrease of 5 logs in all tested acidic conditions. On the other hand, the spores of *B. subtilis* SM10.1 were able to withstand the acidic condition of pH two and showed a slight decrease in viability. The acidic environment is known to cause acidification of cytoplasm, leading to damage in cell structure, macromolecules, and enzymes, thus inhibiting metabolism [13]. However, spores are more resistant to chemical exposure due to its dense coat and the presence of detoxification enzymes in its coat [14].

| pH | Vegetative cells | Spores |
|----|-----------------|--------|
|    | 0 h 1 h 2 h 3 h | 0 h 1 h 2 h 3 h |
| 2  | 7.16 ± 0.09ₐ  1.56 ± 0.24ₐ 1.76 ± 0.45ₐ 1.94 ± 0.30ₐ | 7.36 ± 0.08ₐ  7.15 ± 0.03ₐ  6.71 ± 0.05ₐ  6.60 ± 0.10ₐ |
| 3  | 7.16 ± 0.09ₐ  2.11 ± 1.15ₐ 2.09 ± 0.29ₐ 1.79 ± 0.20ₐ | 7.37 ± 0.03ₐ  4.01 ± 0.41ₐ  4.40 ± 0.13ₐ |
| 4  | 7.20 ± 0.02ₐ  2.23 ± 0.29ₐ 1.96 ± 0.32ₐ 2.12 ± 0.19ₐ | - - - - |

\(a-c\) Values with the same letter in the same row are not significantly different (p > 0.5)

| Bile concentration | Viable cell number (log CFU/ml) |
|--------------------|---------------------------------|
| 0.0%               | 0 h 2 h 4 h                     |
| 0.3%               | 0.5%                            |

\(a-c\) Values with the same letter in the same row are not significantly different (p > 0.5)
Table 2 shows that in the presence of 0.3% and 0.5% bile salts, the number of B. subtilis SM10.1 cells decreased around 2 logs and 3 logs, respectively. Bile salts that act like detergents can damage lipid membranes and macromolecules in bacteria. Tolerance of B. subtilis SM10.1 to bile salts might be possible as the bacteria adapt to stress caused by the bile salts by producing certain proteins or enzymes, e.g., bile salt hydrolase [15].

3.2. Antimicrobial activity

Antimicrobial activity is a desirable property in potential probiotics as it can limit the growth of pathogens in the digestive tract. The cross-streaking method showed that B. subtilis SM10.1 has antimicrobial activity against E. coli and S. aureus. E. coli and S. aureus's growth was inhibited in the area close to B. subtilis SM10.1 (Figure 1). Small colonies of pathogen bacteria were observed at the inhibition zone compared to abundant growth far from B. subtilis SM10.1. E. coli, but not S. aureus, could grow closer to B. subtilis SM10.1, indicating that S. aureus was more susceptible to the antimicrobial activity of B. subtilis SM10.1. Since S. aureus is a Gram-positive bacterium and E. coli is a Gram-negative bacterium; the results implied that the secondary metabolites produced by B. subtilis SM10.1 are more effective toward Gram-positive than Gram-negative bacteria. Secondary metabolites of B. subtilis which act as antimicrobial include variations of ribosomal and non-ribosomal peptides, polyketides, and volatile compounds [16].

Figure 1. Antimicrobial activity of (c) B. subtilis SM10.1 against (a) S. aureus and (b) E. coli.

3.3. Antibiotic susceptibility

Antibiotic susceptibility test was done to identify if B. subtilis SM10.1 is susceptible or resistant to certain antibiotics. Resistance to antibiotics is an undesirable trait for a probiotic because resistance genes might be transferred to the microflora in the gastrointestinal tract through mobile elements, such as plasmid [16]. In this study, antibiotic susceptibility test was done with 23 kinds of antibiotic discs.

Table 3. Antibiotic susceptibility profile of B. subtilis SM10.1

| Antibiotics          | Zone diameter | Interpretation | Antibiotics          | Zone diameter | Interpretation |
|----------------------|---------------|----------------|----------------------|---------------|----------------|
| Ampicillin (10 μg)   | 36 mm         | S              | Nalidixic Acid (30 μg) | 30 mm         | S              |
| Amoxicillin (2 μg)   | 37 mm         | S              | Neomycin (30 μg)     | 19 mm         | M              |
| Bacitracin (10 IU)   | 12 mm         | R              | Ofloxacin (5 μg)     | 37 mm         | S              |
| Cefoxitin (30 μg)    | 41 mm         | S              | Oxacillin (1 μg)     | 27 mm         | S              |
| Chloramphenicol (30 μg) | 43 mm     | S              | Rifampicin (5 μg)    | 23 mm         | S              |
| Ciprofloxacin (5 μg) | 42 mm         | S              | Streptomycin (10 μg) | 10 mm         | R              |
| Clindamycin (2 μg)   | 14 mm         | R              | Sulfonamide (300 μg) | 40 mm         | S              |
| Erythromycin (15 μg) | 26 mm         | S              | Tetracycline (30 μg) | 33 mm         | S              |
| Gentamycin (10 μg)   | 23 mm         | S              | Tiamulin (30 μg)     | 25 mm         | S              |
| Kanamycin (30 μg)    | 27 mm         | S              | Tylosin (30 μg)      | 30 mm         | S              |
| Lincomycin (2 μg)    | N/A           | -              | Vancomycin (30 μg)   | 30 mm         | S              |
| Methicillin (5 μg)   | 37 mm         | S              |                      |               |                |

The interpretation was made according to Gu et al. [10]; S: susceptible (≥ 20 mm); M: moderate susceptible (15-19 mm); R: resistant (≤ 14 mm); N/A: not available.
Table 3 shows that bacitracin, clindamycin, and streptomycin formed the smallest inhibition zones toward \textit{B. subtilis} SM10.1. Meanwhile, the inhibition zone of lincomycin could not be determined. Interpretation of resistance/susceptibility of \textit{B. subtilis} SM 10.1 against antibiotics was made according to the study by [10]. According to the interpretation, \textit{B. subtilis} SM10.1 was categorized as resistant to bacitracin, clindamycin, and streptomycin but susceptible to other antibiotics. However, further study is needed to explore the location of the resistance genes in \textit{B. subtilis} SM10.1. The transferability of antibiotic-resistant genes is more probable for genes on the plasmid while unlikely for genes on the genome [17].

3.4. \textit{Auto-aggregation and co-aggregation}

Auto-aggregation and co-aggregation are associated with the GI tract's potential colonization ability and colony-disrupting properties toward pathogens [18]. For auto-aggregation and co-aggregation using a spectrophotometer, sampling was done by taking the upper part of the bacterial suspension. The aggregate of bacteria, if formed, would go down to the bottom of the tube. Thus, the absorbance of the sample would be lower in comparison to the initial absorbance.

Figure 2 shows that auto-aggregation and co-aggregation of \textit{B. subtilis} SM10.1, \textit{E. coli}, and \textit{S. aureus} seemed to increase over time. After four hours of incubation, \textit{B. subtilis} SM10.1 showed the highest auto-aggregation of > 50%, followed by \textit{S. aureus} and \textit{E. coli} with 30% and 17% aggregation, respectively. Co-aggregation between \textit{B. subtilis} SM10.1 and \textit{E. coli} was slightly higher than \textit{B. subtilis} SM10.1 and \textit{S. aureus}, though the percentages of both co-aggregation were close to 40%.

![Figure 2](image_url)

\textbf{Figure 2.} Auto-aggregation of \textit{B. subtilis} SM10.1 and its co-aggregation with \textit{E. coli} and \textit{S. aureus}

3.5. \textit{Adhesion to epithelial cells}

Adhesion of probiotics to epithelial of GI tract is important to support the survival of probiotics. In this study, human buccal epithelial was used as representative of the epithelial cells in the GI tract. After an hour incubation of human buccal epithelial with \textit{B. subtilis} SM10.1, it was observed that some bacterial cells were attached to epithelial cells (Figure 3). The results suggest that \textit{B. subtilis} SM10.1 might have adhesion properties to epithelial cells. Adhesion to mucus and gastrointestinal epithelial depends on specific macromolecules in the bacterial cell wall, including proteins, polysaccharide, and lipoteichoic acid in Gram-positive bacteria proteins and liposaccharides in Gram-negative bacteria [19]. In probiotics such as \textit{B. cereus}, it was found that S-layer proteins, flagellin, and cell-bound protease affected the good adhesion ability of the bacteria [20]. \textit{B. subtilis} SM10.1, a Gram-positive bacteria, might have macromolecules supporting its adhesion ability. However, further study is needed to confirm any specific macromolecules of \textit{B. subtilis} SM10.1 which are able to interact with mucus and GI epithelial.
Figure 3. Adhesion of *B. subtilis* SM10.1 to (a) epithelial cells; (b) before and (c) after incubation. Arrow indicates bacterial cells that were attached to epithelial cells.

4. Conclusion

The probiotic characterization of *Bacillus subtilis* SM10.1 showed that the vegetative cells of *B. subtilis* SM10.1 were less tolerant to low pH than the spores. However, the vegetative cells could survive at 0.3% and 0.5% bile salts concentration. The antimicrobial activity of *B. subtilis* SM10.1 was successfully detected against *E. coli* and *S. aureus* using the cross-streak method. *B. subtilis* SM10.1 was resistant to streptomycin, bacitracin, and clindamycin. However, resistance to lincomycin has not been determined. *B. subtilis* SM10.1 could form co-aggregation with *E. coli* and *S. aureus*. In addition, *B. subtilis* SM10.1 exhibited the adhesion ability on epithelial cells. Based on the results, it is suggested that *B. subtilis* SM10.1 has the potential to be categorized as probiotic. Nevertheless, further research is needed to confirm the probiotic properties of *B. subtilis* SM10.1.

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