Safety Properties and Probiotic Potential of *Bacillus subtilis* KATMIRA1933 and *Bacillus amyloliquefaciens* B-1895

Ammar AlGburi¹,², Anna Volski³, Carla Cugini⁴, Emily M. Walsh⁵, Vladimir A. Chistyakov⁶, Maria S. Mazanko⁶, Anzhelica B. Bren⁶, Leon M. T. Dicks⁷, Michael L. Chikindas⁸,⁹,¹⁰*

¹Department of Biochemistry and Microbiology, Rutgers State University, New Brunswick, NJ, USA
²Department of Microbiology, Veterinary College, Diyala University, Baqubah, Iraq
³School of Arts and Science, Rutgers State University, New Brunswick, NJ, USA
⁴Department of Oral Biology, Rutgers School of Dental Medicine, Newark, NJ, USA
⁵Department of Plant Biology and Pathology, Rutgers State University, New Brunswick, NJ, USA
⁶D. I. Ivanovsky Academy of Biology and Biotechnology, Southern Federal University, Rostov-on-Don, Russia
⁷Department of Microbiology, University of Stellenbosch, Matieland, South Africa
⁸Health Promoting Naturals Laboratory, School of Environmental and Biological Sciences, Rutgers State University, New Brunswick, NJ, USA
⁹Astrabiol, LLC, Highland Park, NJ, USA
¹⁰Center for Digestive Health, New Jersey Institute for Food, Nutrition and Health, New Brunswick, NJ, USA

Email: tchikindas@aesop.rutgers.edu

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Abstract

This study reports on the safety and putative probiotic properties of *Bacillus amyloliquefaciens* B-1895 and *Bacillus subtilis* KATMIRA1933. According to the bacterial reverse mutation (Ames) test, cell-free supernatants of *B. amyloliquefaciens* B-1895 and *B. subtilis* KATMIRA1933 were not mutagenic. The two strains co-aggregated with *Escherichia coli* and *Pseudomonas aeruginosa*, and cell-free supernatants inhibited the growth of *Streptococcus intermedius* and *Porphyromonas gingivalis*. Endospores of *B. amyloliquefaciens* B-1895 and *B. subtilis* KATMIRA1933 were tolerant to 0.3% (w/v) bile salts and survived incubation for 4 h in MRS broth at pH 2.0 to 3.0. The ability of the two strains to produce antimicrobial compounds potentiates their application in health care formulations, personal care products, food, and animal feed.

*Corresponding author.
Keywords

Bacillus, Safety, Probiotics, Antimicrobials, Bacteriocins, Spores

1. Introduction

According to the World Health Organization, probiotics are “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” [1]. Probiotic strains with the ability to produce antimicrobial compounds are often used to control the growth of pathogenic microorganisms in fermented food and animal feed. The strains may also be incorporated into personal care products [2] [3]. Daily supplementation with probiotics proofed effective in the alleviation of cold and flu symptoms [4], and as food supplement to patients on cancer treatment [5]. Many probiotic bacteria produce a broad range of effective antimicrobials, including lactic acid, hydrogen peroxide and bacteriocins.

Bacteriocins are commonly defined as genetically encoded substances of a proteinaceous nature produced by virtually all bacteria and are active against various, most closely-related, microorganisms [6]. This positions them as a very appealing alternative to antibiotics and chemical stressors, especially in an age when alternative bacterial infection therapies are being widely investigated [7].

Several strains of Bacillus spp. have been recognized as safe for food or industrial applications and, importantly, have been documented as probiotics [8].

This study investigates several characteristics of Bacillus subtilis KATMIRA1933 and Bacillus amyloliquefaciens B-1895, both of which are suspected to have probiotic properties. B. subtilis KATMIRA1933, isolated from a dairy product called Yogu Farm, has been unwittingly consumed by humans for years without harmful effects [9]. The strain produces antimicrobial proteins, including subtilosin A [9]. Genomic analysis has shown that B. amyloliquefaciens B-1895, isolated from soil, has the potential to produce a number of proteolytic enzymes and subtilin, an antimicrobial peptide active against foodborne pathogen Listeria monocytogenes [10]. The strain is used as a probiotic in royal fish [11]. Inclusion of B. amyloliquefaciens B-1895 into bird feed enhanced immunity to the Newcastle virus [12] and improved the body weight of the birds [13]. In addition, fermentates of both strains were reported as having antioxidant and DNA protective activities [14].

Any microorganism considered for use as a probiotic must be tested for specific advantageous characteristics, balanced by a thorough evaluation of its safety. The antibiotic susceptibility of bacterial cells to routinely prescribed antibiotics is essential for the putative probiotic’s safety evaluation. Microbial resistance to antibiotics in part is a gene-encoded mechanism that occurs either by genetic mutation or by gaining resistance via horizontal or vertical gene transfer [15]. It is important to find out if antibiotic-resistance gene(s) are transferable from probiotics to the commensal microorganisms or to pathogenic bacteria. New forms of resistant pathogens may emerge if such genes are transferred from probiotics to pathogens [16]. Many studies have been conducted to identify the antibiotic-resistance genes in Bacillus species [17]-[20].

Some products produced by bacteria have the potential to damage host cells. It is thus important to screen for such products when assessing the safety of a strain. In particular, hemolysin production by bacteria has been identified as a virulence-associated feature [21]. Various bacterial species, including Bacillus cereus and group A streptococci, which produce hemolysin BL and streptolysin-o, respectively, are considered as pathogens due to their potent hemolytic activity [22]. The hemolytic mechanism in Bacillus is not fully understood. However, recent studies have been performed to identify the gene(s) responsible for hemolytic activity [23] [24]. Even though the hemolytic activity of some Bacillus spp., such as Bacillus subtilis, is less than in pathogens [25], these isolates may be considered unsafe for food or personal health care applications until the effect of this virulence factor is either eliminated, modified, or confirmed as causing no harm to the eukaryotic host.

Bacillus species have a long history of use in biotechnology and as dietary supplements for humans and animals of agricultural importance. Also, bacilli have been engineered to produce biologically active substances such as antibiotics and enzymes [26]. A distinctive feature of the Bacillus spp. is high proteolytic activity [27]. The advantages and importance of proteolytic enzymes have been widely reported. Briefly, they include the activation of regeneration processes, the enhancement of normal digestion, and degradation of allergic compounds [27]-[29].
Mutagenicity and carcinogenicity assessments of the antimicrobial substances are the harbinger to efficiently evaluate bacterial products possessing antibacterial activity prior to their use in pharmaceutical applications. The Ames test is used to evaluate the mutagenic potential of substances by determining if the chemical causes DNA damage that leads to genetic mutations in Salmonella spp. Association between mutagens and cancer induction [31] raises the importance of recruiting a screening test for mutagenicity to ensure the safe consumption of such chemicals.

As many probiotics are to be consumed, their selection depends on bacterial tolerance to acids and bile salts. Candidacy of a probiotic depends on the ability of bacterial cells or their spores to survive and grow at the high acidity (pH 3 or below) of the stomach [32] and with the detergent-like activity of intestinal bile salts that disrupt the cellular membrane [33]. In vitro, the tolerance of Bacillus species to acids and bile salts reflects their survival rates and viability through the gastrointestinal tract [32] [34].

Coaggregation is the adherence of genetically distinct bacteria and is considered as a desirable characteristic in a probiotic microorganism [35]. It is believed to facilitate the integration of exogenous bacteria, which is important for the development of multispecies biofilms [35]. Coaggregation allows a beneficial organism to adhere to a pathogenic organism. Aggregation may also help the bacterium adhere to different surfaces, which is very relevant to human health. Adhesion may also allow probiotic organisms to create a barrier, which could effectively prevent colonization by pathogens [36]. Auto-aggregation of probiotic strains may be required for adhesion to intestinal epithelial cells, which would keep them from being flushed out by the body, and would in such a way give them an advantage over other organisms [36]. Though not all mechanisms of action have been determined, further research can reveal the more complex aspects and implications of this ability.

The objective of this study is to investigate whether B. amyloliquefaciens B-1895 and B. subtilis KATMIRA 1933 possess additional health benefits that would qualify them to be probiotic candidates. A battery of tests is commonly employed and has been addressed in the study on B. subtilis KATMIRA1933 and B. amyloliquefaciens B-1895. Antibiotic susceptibility, hemolytic/fibrinolytic activity, proteolytic activity, bacterial reverse mutation, tolerance to acids and bile salts, and bacterial auto-aggregate/co-aggregate abilities were evaluated and analyzed for both bacilli. The antimicrobial activity of Cell-Free Supernatants (CFSs) of tested bacilli against pathogens was also evaluated. Regarding the ability to auto-aggregate and co-aggregate, the most appropriate method for the mathematical interpretation of collected data was identified. A visual analysis utilizing microscopy was used as a mode of comparison for evaluating two methods of mathematical analysis. It was determined that both the extracts possessed unique antibacterial capabilities along with the desirable traits that would allow them to progress as candidates for probiotics therapies.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

Frozen stocks of B. subtilis KATMIRA1933 and B. amyloliquefaciens B-1895 were inoculated in De Man, Rogosa and Sharpe (MRS) medium (Becton Dickinson and Company, Sparks, Maryland, USA) and incubated aerobically with shaking at 250 rpm for 24 h at 37°C. M. luteus ATCC10240 was used as a reference microorganism. The pathogenic bacteria included in our study are listed in Table 1. These bacterial strains were inoculated into tryptic soy medium (Becton Dickinson and Company, Sparks, Maryland, USA) and incubated aerobically for 24 h at 37°C. The oral pathogens, upon revival from ~80°C DMSO stocks, were maintained on trypticase soy broth (TSB) and agar plates containing haemin 1 µg mL⁻¹, menadione 1 µg mL⁻¹, 20% defibrinated sheep’s blood (BAPHK) at 37°C under anaerobic conditions (5% H₂, 10% CO₂, 85% N₂). S. intermedius strain F0413 was maintained on Brain Heart Infusion broth at 37°C under anaerobic conditions, while S. mutans strain 25,175 was incubated aerobically. Broth cultures of Porphyromonas gingivalis strains, Prevotella intermedia strains, and Fusobacterium nucleatum were grown in Todd–Hewitt broth (THB) containing haemin 1 µg mL⁻¹ and menadione 1 µg mL⁻¹ (designated THBHK) at 37°C under anaerobic conditions.

2.2. Antimicrobial Activity of CFS of Studied Bacillus Strains

Cell free supernatant of two tested bacilli strains were prepared as previously described by Sutyak et al. [9]. Broth cultures (1.5 mL) of oral pathogens were grown for 24 h in the appropriate atmosphere and media. Culture density for each strain was determined using OD₆₀₀ values. All strains were adjusted to an OD₆₀₀ of 0.1 to correspond to 1 × 10⁶ CFU·mL⁻¹. Cultures were swabbed to the appropriate solid support agar media, which had
Table 1. Bacterial strains and culture conditions used in this study.

| Bacterial Species and Origin | Source/Culture Medium | Importance | References |
|------------------------------|------------------------|------------|------------|
| *Bacillus subtilis* KATMIRA1933 | Dairy products/MRS | Bacteriocin-producer bacteria | [9] |
| *Bacillus amyloliquefaciens* B-1895 | Soil isolate/MRS | Proteolytic enzyme-producer Probiotics in royal fish Food supplement | [11] [12] [50] |
| *Micrococcus luteus* ATCC10240 | Skin of humans and other animals and in soil, marine and fresh water, plants, fomites, dust, and air/TSB | Opportunistic pathogens for the immunocompromised | [89] [90] |
| *Escherichia coli* O157:H7 | Food and human pathogen/TSB | Intestinal infection food-related outbreaks | [91] |
| *Staphylococcus aureus* ATCC 13565 | Food and human pathogen/TSB | Staphylococcal food poisoning, nosocomial infections and infections on indwelling medical devices | [92] [93] |
| *Streptococcus mutans* ATCC 33402 and *Streptococcus mutans* strain 25175 | Human-Oral pathogen/BHI | Dental decay | [94] |
| *Streptococcus intermedius* strain F0413 | Human CNS’ and pulmonary pathogen/BHI | CNS abscesses pulmonary infection | [95] [96] |
| *Salmonella enterica* Stanley 7308 | Intestinal pathogen/TSB | Contaminated seeds sprout-bacteria | [97] [98] |
| *Listeria monocytogenes* Scott A | Foodborne and human pathogen/TSB | Food-borne and human disease (human Listeriosis) | [99] [100] |
| *Campylobacter jejuni* ATCC33560 | Human and nimal pathogen/TSB | Gastroenteritis (zoonotic infection) | [101] [102] |
| *Pseudomonas aeruginosa* ATCC15442 | Human pathogen/TSB | Contamination of ophthalmic pharmaceuticals and nosocomial pathogen (cystic fibrosis) | [103]-[105] |
| *Porphyromonas gingivalis* strains 381, W83, 33277, A7A1-28 (ATCC 53977) | Human-oral pathogen/TSB-THB | Periodontal breakdown and disease and acute necrotizing ulcerative gingivitis | [106]-[108] |
| *Prevotella intermedia* strains 25611, 17 | Human-oral pathogen/TSB-THB | Development and progression of periodontal disease | [109] [110] |
| *Fusobacterium nucleatum* strain ATCC 25586 | Human commensal and pathogen/TSB-THB | Oral infections, adverse pregnancy outcomes, GI& disorders, colorectal cancer | [111]-[114] |

*CNS: Central Nervous system, GI: Gastrointestinal.

been dried for 20 min in a tissue culture hood. Plates that were to be used under anaerobic conditions were placed in the chamber and all further steps were carried out within. Using the wide end of a 200 µL yellow pipette tip, holes were bored into the media, creating a well to accommodate supernatants. Cell-free supernatant (120 µL) from *B. subtilis* KATMIRA1933 and B-1895 were added to each well, in triplicate. Plates were incubated for 5 days at 37°C under anaerobic conditions and the diameter of the zones of inhibition were measured in millimeters (mm) with a digital caliper.

2.3. Antibiotic Susceptibility of Studied Bacilli

*B. subtilis* KATMIRA1933, *B. amyloliquefaciens* B-1895, and *M. luteus* ATCC 10,240 were included in this assay. Bacterial strains grown overnight were diluted 1:100 with corresponding fresh media to yield approximately 10⁶ CFU·mL⁻¹. This was verified by the plate counting method. The disc diffusion test was conducted according to the (CLSI) Performance Standards for Antimicrobial Disk Susceptibility Tests [37]. The tested antimicrobial discs included ampicillin (10 µg), erythromycin (15 µg), and tetracycline (30 µg) from Becton Dickinson and Company (Sparks, Maryland, USA), while bacitracin (10 µg), chloramphenicol (30 µg), kanamycin (30 µg), penicillin (10 IU), streptomycin (10 µg) and oxytetracycline (30 µg) were from Benex Limited (Shannon, Co., Clare). Plates were incubated for 24 h at 37°C. Radii of zones of inhibition were measured in millimeters (mm) with a digital caliper (Fischer Scientific, Pittsburg, PA, USA) from the edge of the disk to the
edge of the inhibition zone.

2.4. Bacterial Reverse Mutation (Ames) Assay

The mutagenicity assessment of subtilosin, CFSs of *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 was carried out according to Maron and Ames [38], Cappuccino and Sherman and [39] with minor modifications. Briefly, *Salmonella typhimurium* K-6 TA1535 overnight growth was prepared according to the manual a day before performing experiment. Top agars were melted using a hot water bath (45°C). Then, 300 µL of histidine/biotin and 100 µL of the overnight culture of *S. typhimurium* K-6 TA1535 were added to the top agars. Top agar contents were gently mixed and immediately poured over the minimal agar plates. A sterile forceps was used to pick up a filter paper disc and dip it into the two microcentrifuge tubes containing different concentrations of tested samples. Positive and negative controls were included. After filter paper discs were saturated with tested chemicals, they were placed into the center of a minimal agar plate that was laid out with top agar containing biotin and bacterial growth suspension. All the plates were incubated aerobically at 37°C for 48 - 72 h in an inverted position. The results were evaluated by counting the number of colonies that grew on the agar plates. The mutant frequency was calculated for tested samples. Mutant frequency was calculated as the number of revertant colonies in treated plates divided by their numbers in the negative control.

\[ MF = \frac{NT}{NC} \]

where \( MF \) = Mutant Frequency, \( NT \) = Number of revertant colonies on treated plates, \( NC \) = Numbers of colonies on negative control plate.

The results were expressed as following: the substance considered has mutagenic activity when its MF value is \( \geq 2 \), is a possible mutagen when the value ranges of 1.7 to 1.9, and has no mutagenic activity when the frequency ranges \( \leq 1 - 1.6 \) [40].

2.5. Determination of Protease Activity by the "Stabbing" Method

Detection of the proteolytic activity of *Bacillus* strains, *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895, was performed as described by Ponmurugan [41] with minor modifications. Briefly, from the frozen stocks, bacilli were maintained in MRS broth aerobically with agitation (150 rpm), for 24 h at 37°C. From the last overnight culture, 10 µL was spread on MRS agar by streaking with a loop and plates were incubated aerobically for 24 h at 37°C. After the incubation period, one colony was picked up using an inoculating loop and stabbed into a milk agar plate. A milk agar composed of peptone (0.1%), NaCl (0.5%) and skim milk (10%) was prepared according to Uyar *et al.* [42] with some minor modifications. The components of the milk agar medium were mixed thoroughly with double deionized water and autoclaved for 15 min. The inoculated plates were incubated for 24 h at 37°C under aerobic conditions. The results were reported as following: a clear zone of proteolytic activity around inoculated colonies represented protease positive and if the clear zone did not appear, it was protease negative.

2.6. Hemolytic Activity on Whole and Defibrinated Blood Agar

A hemolytic assay was performed as described by Luo *et al.* [43] with minor modifications. Instead of inoculating blood agar with 10 µL of bacterial suspension using a disposable loop, a polyester tipped applicator (Fischer scientific, Pittsburg, PA) was used to spot-inoculate onto the whole and defibrinated blood agar. This was achieved by touching the tip of the applicator to one bacterial colony before using the tip to lightly touch the fresh blood agar while rotating the applicator. By using the polyester applicators, circular inoculation sites of about 5 mm in diameter were formed. Each bacterial strain was inoculated onto the blood agar using this method, and sufficient space was given between each spot. Inoculated blood agar plates were then incubated aerobically for 24 h at 37°C. Plates were then checked for hemolytic activity.

2.7. Fibrinolytic Activity Test

One milliliter of LB broth was inoculated with *B. amyloliquefaciens* B-1895 and *B. subtilis* KATMIRA1933 and incubated for 18 h at 37°C with agitation. Each culture was adjusted to one unit of the MacFarland Turbidity
standard. Then, 1 mL of the adjusted cell suspensions and 24 mL of fresh LB were mixed in 50 mL culture flasks, and incubated 5 days at 37°C. The CFSs were collected by centrifugation (4480 g, 15 min, and 4°C). Plates containing fibrin were prepared accordingly, the 1.5% agar-based formulation (10 mL) consisting of 0.4% fibrinogen in 50 mM sodium phosphate buffer at pH 7.4 was mixed with 0.1 mL of thrombin (10 NIH units) poured into the Petri dishes (15 mL), and left to polymerize and to dry at room temperature. Then, 7 mm diameter holes were punched in the solidified agar. These holes were filled with 30 μL of the CFSs and incubated overnight. The clear zones of fibrinolytic activity were measured with a digital caliper.

2.8. Coaggregation Test

The coaggregation assay was performed to evaluate the coaggregation ability of B. subtilis KATMIRA1933 and B. amyloliquefaciens B-1895 strains with select pathogens following the method described by Cisar et al. [44] with some modifications. Bacterial cultures were harvested from the planktonically grown cells incubated at 37°C by centrifugation (4480 g, 15 min, 23°C); they were washed with sterile phosphate buffered saline (PBS) twice. After the second wash, the harvested cells were re-suspended in PBS and the optical density (OD 600) was adjusted to 0.25. In a 96-well microtiter plate, 100 µL of each test strain was mixed with 100 µL of Bacillus strain, while 200 µL of each bacterial suspension in monoculture was used as controls. The plate was placed in a micro-titer plate spectrophotometric reader (SmartSpec™ 3000) and kept at 30°C. Measurements of OD 600 were taken once per hour for 24 h and calculated for coaggregation. Each experiment was performed in quadruplicate. Samples of 100 µL were taken after 2 h reading for Gram staining and observed microscopically for coaggregation (Figure 1).

2.9. Mathematical Analyses

Two methods were employed to calculate the percent of coaggregation after 2 h incubation in each of the mixtures, for the purpose of comparison. In the first method, the percent auto-aggregation of each bacterium and the percent coaggregation in each mixture was calculated as described by Ledder et al. [35] with the following equation:

Method 1:

\[ \text{Coaggregation} \% = \left( \frac{x - y}{x} \right) \times 100 \]

where \(x\) is the pre-incubation value and \(y\) is the post-incubation value at a certain time point.

The second method employed the equation described by Handley et al. [45]:

Method 2:

\[ \text{Coaggregation} \% = \left[ 1 - \frac{2(x + y)}{(Ax + Ay)} \right] \times 100 \]

where \(Ax\) and \(Ay\) are the organisms as controls and \((x + y)\) is a mixture of the two.

Both methods used optical density data obtained after 2 h, at OD 600. The data from the two methods were compared and the more appropriate method was chosen from the analysis of 2 h and used for the 8 h analysis.

2.10. Microscopy

Bacterial interactions at the 2 h time points were visualized on slides using histological techniques. To visualize, bacteria were stained with Gram stain (BD, Becton and Company, Maryland, US). Images were obtained with a Nikon DS-Fi1 camera mounted on a Nikon Eclipse 80i compound microscope using the 100×/1.25 oil objective. Images were analyzed using Nikon, NIS- Elements D3.0 software. The amount of coaggregation was visually analyzed and scored with a scoring system, with 0 being the absence of coaggregation and 4 being an abundance of coaggregation (Figure 1).

2.11. Production of Bacillus Spores

Sporulation-inducing esculin agar was composed of esculin hydrate (E8250-5G) (Sigma-Aldrich), 1 g, ferric
Figure 1. Microscopic analysis of auto-aggregation and coaggregation. A 0-4 scoring system was utilized, with 0 representing no adhesion between similar or different microorganisms, and 4 representing maximum aggregation after 2 h incubation.

citrate (Fisher scientific), 0.5 g, and BHI, 40 g. These components were mixed and completely dissolved in deionized water up to 1 L. The pH of the mixture was adjusted to 7.0, agar (1.5%) was added and the medium then was autoclaved. *Bacillus* sporulation was achieved following Franklin and Clark [46] with minor modifications. Briefly, the esculin agar medium was poured into 150 × 15 mm Petri dishes to achieve about a 10 mm thick layer. *Bacillus* grown on MRS plate was scraped and seeded onto an esculin plate. The inoculated agar was incubated for 15 - 25 days aerobically at 37°C. After the first five days of incubation, spore production by the *Bacillus* strains was monitored daily using light microscope. Once sufficient numbers of spores were produced in the grown colonies, they were harvested using sterile inoculating loops. The spores were washed with sterile distilled water and pelleted by centrifugation (5444.5 g, 20 min at 4°C). The pellets were re-suspended with 20 mL sterile distilled water, glass beads were added, and incubated with agitation at 75°C - 80°C for 25 min to ensure the killing of the vegetative cells. Following that, tubes were placed on ice for 10 min and then the glass beads were discarded. The suspension was collected by centrifugation (5444.5 g, for 20 min, at 4°C) and washed three times with ice-cold sterile water. The spores were re-suspended in a minimum volume of sterile ice-cold water and counted by plating. The spore suspension was aliquoted and stored at −80°C for future use.

2.12. Acid and Bile Tolerance of *Bacillus* Spores

The acid and bile tolerance method was performed according to Hyronimus et al. [47] with minor modifications. Briefly, a frozen stock of *Bacillus* spores was diluted with PBS to achieve 10^8 spores·mL⁻¹. Tubes containing spores were incubated at 80°C for 20 min with agitation to get rid of remaining vegetative cells. After heat treatment, the tubes were placed on ice for 10 min. Ten milliliters of MRS broth was transferred into sterile tubes and the broth pH was adjusted to different pH values: 2.0, 2.5, 3.0 using 0.1 N HCl (Sigma Aldrich, St.
Louis, MO). Ten milliliters of MRS broth containing 0.3% bile salts (Oxoid Ltd., Basingstoke, UK) was prepared and transferred into 50 mL test tubes. Control tubes containing broth medium only without adding acid or bile salts were included in this experiment. A 100 µL of diluted spore suspension containing 5 × 10⁷ Spore Forming Unit per milliliter (SFU·mL⁻¹) was dispensed into each tube (acid, bile, and control tubes). At each time interval, 0, 0.5, 1, 2, and 4 h, the spread-plate method was used to enumerate the numbers of surviving spores after acid and bile treatment on MRS plates. The inoculated plates were incubated aerobically at 37°C for 24 - 30 h. For each treatment, the survival rates were measured. Survival rate was defined as the percentages of the logarithmic number of SFU at each time point (s) divided by SFU numbers at 0 time point (control). For example, the survival rates after 4 h is \( \frac{\log_{10} \text{SFU·mL}^{-1} \text{at } 4 \text{ h}}{\log_{10} \text{SFU·mL}^{-1} \text{at } 0 \text{ h}} \times 100 \).

2.1.3. Statistical Analysis

For the antibiotic susceptibility assay and antimicrobial activity of CFS of studied *Bacillus* strains, experiments were performed at least three times in triplicate. Co-aggregation and auto-aggregation experiments were conducted three times; the collected values were then analyzed mathematically and visually. Acid and bile tolerance studies were repeated three times in duplicate and the results shown were expressed as mean (%) ± SD. Student’s *t*-test with two-tailed distribution (Excel, Microsoft Corporation, US) was used to compare the survival rates (%) of the two studied bacilli strains in the 3 sets of pH during the 4 h incubation.

3. Results

3.1. Antimicrobial Activity of CFS of the Studied Bacilli

Selected pathogens were tested for their sensitivity to the CFS of *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 strains (Table 2). All *P. gingivalis* isolates were sensitive to *B. subtilis* KATMIRA1933 CFS, as indicated by a zone of clearing of the bacteria (Table 2). *S. intermedius* was susceptible to CFS of *B. amyloliquefaciens* B-1895 (Table 2). *P. intermedia*, *F. nucleatum* and *S. mutans* were not inhibited by the CFS at the concentrations tested (data not shown).

3.2. Antibiotic Susceptibility of Bacillus Strains

The sensitivity of the *Bacillus* strains to nine antibiotics was assessed according to CLSI [48]. The antibiotics were ampicillin 10 µg, erythromycin 15 µg, tetracycline 30 µg, bacitracin 10 µg, chloramphenicol 30 µg, kanamycin 30 µg, streptomycin 10 µg, oxytetracycline 30 µg, and penicillin 10 IU. Antibiotic susceptibility test results revealed that *B. subtilis* KATMIRA1933 was tolerant to bacitracin and streptomycin, and more susceptible to penicillin, ampicillin and chloramphenicol than other tested antibiotics. The tolerance of *B. amyloliquefaciens* B-1895 strain to bacitracin, streptomycin, tetracycline and oxytetracycline was more than other antibiotics, and its susceptibility to ampicillin and chloramphenicol was the highest. Antibiotic susceptibility of *Bacillus* strains was compared to that of *M. luteus*, which is a frequently used Gram-positive reference bacterium (Table 3).

3.3. Hemolytic and Fibrinolytic Activity Test

Hemolytic activity of the *Bacillus* strains was determined using MRS medium, supplemented by whole blood and MRS, supplemented with defibrinated blood. While *B. amyloliquefaciens* B-1895 produced weak hemolysis on whole blood agar plates, a zone of clearance was observed with *B. subtilis* KATMIRA1933 (Table 4). On defibrinated blood agar, both *Bacillus* strains displayed no hemolytic activity. *S. aureus* and *M. luteus* were used as positive and negative controls, respectively, for this set of experiments. Because hemolytic activity of *B. subtilis* KATMIRA1933 was observed on whole blood agar, we hypothesized that the predominant is the fibrinolytic activity. Using a fibrinolytic activity assay, it was demonstrated that *B. subtilis* KATMIRA1933 CFS produced a fibrinolytic zone of 14 mm diameter, while *B. amyloliquefaciens* B-1895 CFS formed a zone of 11 mm diameter.

3.4. Proteolytic Activity Test

To determine whether the *Bacillus* strains possess proteolytic activity, the presence or absence of a zone of clearance around bacterial growth and/or bacterial CFS on milk agar was determined; clearance is indicative of
Table 2. Bacillus extract-induced zones of inhibition of oral pathogens.

|                                 | Bacillus amyloliquefaciens B-1895 Extract | Bacillus subtilis KATMIRA1933 Extract |
|---------------------------------|------------------------------------------|---------------------------------------|
| Dose (µg)                       | Diameter (mm)                             | Diameter (mm)                          |
| Streptococcus intermedius F0413 | 14.8 ± 2.0                                | 15.8 ± 0.6                             |
| Porphyromonas gingivalis 381    |                                          | 15.6 ± 0.6                             |
| Porphyromonas gingivalis 33277  |                                          |                                       |
| Porphyromonas gingivalis W83    |                                          | 13.7 ± 1.0                             |
| Porphyromonas gingivalis A7A1-28|                                          | 13.6 ± 0.9                             |

Table 3. Antibiotic susceptibility test of Bacillus subtilis KATMIRA1933 and Bacillus amyloliquefaciens B-1895.

| Antimicrobial Disc | Dose (µg) | Bacillus subtilis KATMIRA1933 | Bacillus amyloliquefaciens B-1895 | Micrococcus luteus ATTCC1420 |
|-------------------|-----------|-------------------------------|-----------------------------------|-------------------------------|
| Ampicillin        | 10        | 16 ± 0.4 mm                   | 18 ± 0.3 mm                       | 14 ± 0.4 mm                   |
| Erythromycin      | 15        | 15 ± 0.2 mm                   | 15 ± 0.4 mm                       | 15 ± 0.2 mm                   |
| Tetracycline      | 30        | 13 ± 0.6 mm                   | 8 ± 0.2 mm                        | 14 ± 0.9 mm                   |
| Bacitracin        | 10        | 2 ± 0.3 mm                    | 6 ± 0.2 mm                        | 18 ± 0.3 mm                   |
| Chloramphenicol   | 30        | 16 ± 0.3 mm                   | 17 ± 0.4 mm                       | 13 ± 0.4 mm                   |
| Kanamycin         | 30        | 13 ± 0.3 mm                   | 11 ± 0.7 mm                       | 7 ± 0.4 mm                    |
| Penicillin*       | 10        | 17 ± 0.4 mm                   | 15 ± 0.4 mm                       | 14 ± 0.7 mm                   |
| Streptomycin      | 10        | 2 ± 0.7 mm                    | 7 ± 0.3 mm                        | 7 ± 0.4 mm                    |
| Oxytetracycline   | 30        | 14 ± 0.6 mm                   | 9 ± 0.3 mm                        | 15 ± 0.3 mm                   |

*Penicillin was 10 IU.

Table 4. Hemolytic activity of Bacillus strain and their Cell Free Supernatants (CFS).

| Bacterial Species                              | Blood Hemolysis (Whole Blood) | Blood Hemolysis (Defibrinated Blood) |
|-----------------------------------------------|------------------------------|--------------------------------------|
| Micrococcus luteus                            | –                            | –                                    |
| Staphylococcus aureus                         | ++                           | ++                                   |
| Bacillus subtilis KATMIRA1933                 | ++                           | –                                    |
| Bacillus amyloliquefaciens B-1895             | +                            | –                                    |

++ = Complete β-hemolysis, + = Weak hemolysis, – = no hemolysis.

protein hydrolysis. B. subtilis KATMIRA1933 and B. amyloliquefaciens B-1895 cells or CFS showed proteolytic activity with clear zone areas of 13 ± 0.5/5 ± 0.3 mm and 15 ± 0.6/3 ± 0.3 mm, respectively, after 24 h incubation. E. coli O157:H7 and its CFS were used as negative controls (data not shown).

3.5. Bacterial Reversal Mutation (Ames) Assay

To evaluate the mutagenic potential of the Bacillus extracts and purified compounds, with the hope of ensuring these natural products were free of mutagenic factors, the Ames test was conducted using S. typhimurium strain TA1535. In addition to the isolated subtilosin, CFS of the Bacillus strains was tested. The number of revertant colonies was counted on glucose minimal agar and the mutant frequency (MF) was calculated. The number of revertant colonies when MRS broth (negative control) was tested were 15 - 16 CFU per plate (Table 5); similar results were obtained when phosphate buffer saline (PBS; negative control) was tested (18 - 20 CFU per plate) (Table 5). According to Kirkland [40], a frequency that ranges from ≤ 1 - 1.6 for the tested substances indicates no mutagenic activity. The mutant frequency of 50 µg·mL⁻¹ subtilosin was 1.4 higher when compared with concentration of 100 µg·mL⁻¹ and 550 µg·mL⁻¹ (Table 5). The MF of 100%, 50%, 25% and 12.5% CFS of B.
3.6. Auto-Aggregation and Coaggregation of Bacterial Strains

Kinetic measurements of auto-aggregation and coaggregation of the bacilli with pathogenic bacteria was determined during an 8 h time period using an automated micro-titer plate reader to quantitatively evaluate aggregation efficiency. Bacterial strains varied with respect to the time required to observe a high aggregation value. Two methods were used to evaluate the auto-aggregation and coaggregation percentages (Table 6). The highest percentages were obtained using the calculation of method 1, which was previously described by Ledder et al. (2010). Method 1 was chosen in this study as the more convincible method, compared with method 2, after mathematical interpretation of optical density data; the method was similar inits application and more closely matched conclusions made from the microscopic analysis (Figure 1 and Table 6). The percentage of auto-aggregation of *B. amyloliquefaciens* B-1895 was the highest (89.5%), while the lowest was of *S. aureus* and *S. enterica* (14.3% and 15.4%), respectively (Table 7). *B. amyloliquefaciens* B-1895 strain was highly co-aggregative with *E. coli* (47.1%), *P. aeruginosa* (46.9%), *S. enterica* (43.9%) and *L. monocytogenes* (41.9%), but it poorly co-aggregated with *S. aureus* (29.9%). In the case of *B. subtilis* KATMIRA1933, the high coaggregation percentage was observed with *E. coli* (50.3%) followed by *P. aeruginosa* (49.7%), *L. monocytogenes* (48.2%) and *S. enterica* (47.4%), while low coaggregation was observed with *S. aureus* (34%) and *S. mutans* (31.8%) (Table 8).

3.7. Tolerance of *Bacillus* Spores and Vegetative Cells to Acids and Bile Salts

To evaluate the acid and bile salt tolerance of the spores and vegetative cells, the bacilli were exposed to a range of acid (pH 2.0, 2.5 and 3.0) and 0.3% bile salts during 4 h. The survival rates (SR) (%) of *B. subtilis* KATMIRA1933 spores were constant (unchanged) during the incubation period all pHs tested. At pH 2.0, the final SR was 98.4% ± 2.3%, and slightly higher in pH 2.5 and 3.0, which were 98.75% ± 1.76% and 99.15% ± 1.2%, respectively. The 4 h SRs for *B. amyloliquefaciens* B-1895 spores similarly were nearly constant across all pH conditions; in the pH 2.0 and 3.0 environment; the SR was 96.45% ± 5.02%, while at pH 2.5 it was 97.3% ± 3.81%. No significant difference was found in the survival rates of both *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 into the three sets of pH values for 4 h. Bile tolerance of the *Bacillus* spores was 88% ± 1.27% for *B. subtilis* KATMIRA1933 and 84.85% ± 2.05% for *B. amyloliquefaciens* B-1895.
Table 6. Comparison between method 1 (as described by Ledder et al. [35]) and method 2 (as described by Handley et al. [45]) to calculate the coaggregation values of *Bacillus* strains with tested pathogens after 2 h incubation.

| Bacillus Strains          | Bacterial Species              | Method 1 (%) | Method 2 (%) | Microscopic Analysis |
|---------------------------|--------------------------------|--------------|--------------|----------------------|
| *Bacillus subtilis*       | *Escherichia coli*             | 16.5         | 8.1          | 3                    |
| KATMIRA1933               | *Pseudomonas aeruginosa*       | 15.6         | 6.5          | 3                    |
|                           | *Staphylococcus aureus*        | 14.4         | 5.8          | 1 - 2                |
|                           | *Listeria monocytogenes*       | 14.6         | 1.8          | 2 - 3                |
|                           | *Salmonella enterica*          | 17.1         | 3.6          | 3 - 4                |
|                           | *Streptococcus mutans*         | 12           | 1.8          | 2                    |
|                           | *Campylobacter jejuni*         | 14.5         | 1.9          | 2                    |
|                           | *Escherichia coli*             | 31.4         | 5.9          | 3                    |
|                           | *Pseudomonas aeruginosa*       | 31.5         | <0           | 3 - 4                |
|                           | *Staphylococcus aureus*        | 29.9         | <0           | 1                    |
|                           | *Listeria monocytogenes*       | 30.3         | <0           | 3                    |
|                           | *Salmonella enterica*          | 31.6         | <0           | 2                    |
|                           | *Streptococcus mutans*         | 28.9         | <0           | 3                    |
|                           | *Campylobacter jejuni*         | 25.5         | <0           | 2                    |
| *Bacillus amyloliquefaciens* | *Escherichia coli*             |              |              |                      |
| B-1895                    | *Pseudomonas aeruginosa*       |              |              |                      |
|                           | *Staphylococcus aureus*        |              |              |                      |
|                           | *Listeria monocytogenes*       |              |              |                      |
|                           | *Salmonella enterica*          |              |              |                      |
|                           | *Streptococcus mutans*         |              |              |                      |
|                           | *Campylobacter jejuni*         |              |              |                      |

Table 7. Highest auto-aggregation of the tested microorganisms as observed during 8 h of incubation.

| Bacterial Strains          | Auto-Aggregation (%) | Time (h) |
|---------------------------|----------------------|----------|
| *Listeria monocytogenes*  | 28.08                | 6        |
| *Streptococcus mutans*    | 29.4                 | 8        |
| *Escherichia coli*        | 32.8                 | 8        |
| *Staphylococcus aureus*   | 14.3                 | 4        |
| *Pseudomonas aeruginosa*  | 20.5                 | 8        |
| *Salmonella enterica*     | 15.4                 | 8        |
| *Campylobacter jejuni*    | 64.9                 | 8        |
| *Bacillus subtilis*       | 59.5                 | 8        |
| KATMIRA1933               |                      |          |
| *Bacillus amyloliquefaciens* | B-1895              | 89.5     | 8        |

Table 8. The highest co-aggregation % of *Bacillus subtilis* KATMIRA1933 and *Bacillus amyloliquefaciens* B-1895 with the selected pathogens during 8 h of incubation.

| Pathogens                  | *Bacillus subtilis* KATMIRA1933 Cells % | Time (h) | *Bacillus amyloliquefaciens* B-1895 Cells % | Time (h) |
|----------------------------|----------------------------------------|----------|---------------------------------------------|----------|
| *Escherichia coli*         | 50.3                                   | 8        | 47.1                                        | 6        |
| *Pseudomonas aeruginosa*   | 49.7                                   | 8        | 46.9                                        | 8        |
| *Salmonella enterica*      | 47.4                                   | 8        | 43.9                                        | 7        |
| *Campylobacter jejuni*     | 38.7                                   | 8        | 33.3                                        | 4        |
| *Staphylococcus aureus*    | 34                                     | 8        | 29.9                                        | 2        |
| *Streptococcus mutans*     | 31.8                                   | 8        | 35.5                                        | 4        |
| *Listeria monocytogenes*   | 48.2                                   | 8        | 41.9                                        | 5        |
As expected, the tolerance of vegetative cells of *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 strains to acidity was much less than those of the spores. In the case of the *B. subtilis* KATMIRA1933 strain, vegetative cells were incapable of surviving at pH 2.0 and pH 2.5 after 1 h incubation, however at pH 3.0 the SR was 23.9% at 4 h. *B. amyloliquefaciens* B-1895 vegetative cells were more tolerant to acidic conditions than those of *B. subtilis* KATMIRA1933. About 30% of vegetative cells of *B. amyloliquefaciens* B-1895 were able to survive after 4 h at MRS with the different pH values. There was no difference in the tolerance of vegetative cells of studied Bacillus strains to bile salt when compared with the tolerance of their spores (Data not shown). Statistically significant differences in acid tolerance between the *Bacillus* strains were observed after 30 min of incubation (Data not shown). While there was not a statistically significant difference between the vegetative cells of both *Bacillus* strains (P > 0.05), the survival rates (%) in 0.3% bile salts exhibited a statistically significant difference between the spores of the two bacilli strains during the 4 h incubation (P < 0.05) (Data not shown).

4. Discussion

The *B. subtilis* KATMIRA1933 strain was used as a source of subtilosin A and studied for various applications such as for control of food-borne and other human pathogens [9]. *B. amyloliquefaciens* B-1895 strain was reported as a putative probiotic in poultry and fish [13]. Based on the beneficial properties of both microorganisms, we hypothesize on their possible use as probiotics for human and/or agricultural applications. Therefore, the effort was made to look into these strains’ safety and some of their probiotic-related capacities.

Positive health effects were noticed such as increment food consumption and increasing of broilers’ body weight [13]. An association between *Bacillus*-food supplements and immune system stimulation was identified in chicken. When birds were vaccinated against the Newcastle virus, the antibody titers in chicken with a *B. subtilis*-direct feed were greater than those in the control group without bacilli supplement-food [12].

The soil isolate, *B. amyloliquefaciens* B-1895 showed probiotic potential in Azov-Chernomoskaya royal fish by eliminating pathogens and increasing survival rate of fish [11]. Using the RAST server [49], the protein-encoding genes that were responsible for bacitracin-like antibiotics biosynthesis were identified [50].

*B. subtilis* KATMIRA1933 was isolated from a dairy product [9] and it has been extensively consumed by people without any negative reported side effects. *B. subtilis* KATMIRA1933 was detected as an antimicrobial-producer strain. The bacteriocin subtilosin, which was a secondary metabolite of *B. subtilis* KATMIRA1933, showed selective antimicrobial activity against pathogens such as *Gardnerella vaginalis* but not against protective (beneficial) lactobacilli [51] [52].

The antimicrobial activity of CFSs of *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 against oral pathogens was reported in this study. These results attracted our attention moving toward evaluating the probiotic potentials of these bacilli. Safety tests were performed to ensure the innocuous side effects of the utilization of such microorganisms in food and medical applications.

An active area of oral health research has been aimed at the identification of natural products to either kill or attenuate pathogenicity of the key bacterial strains that contribute to oral infections such as caries and periodontal disease. Much of the effort has focused on finding plant-based compounds that are able to act as antimicrobials or bacteria-modulating compounds. An alternative active area of research is the identification of bacterial factors that can act as therapeutics, and as presented here, products of soil isolates that display antimicrobial properties against two classes of sub-gingival plaque organisms that have been associated with periodontitis and second-site infections such as brain or liver abscess, as in the case of *S. intermedius*.

As demonstrated here, *B. subtilis* KATMIRA1933 extract has anti-microbial effects against all *P. gingivalis* strains tests; these represent a combination of fimbrinated, afimbribinated, encapsulated, and non-encapsulated strains. Previous studies addressing *P. gingivalis* susceptibility to *Bacillus* extracts identified that only those without a capsule were susceptible, however, the researchers used different growth conditions which were presented here [53]. In our experience, *P. gingivalis* growth in liquid culture is suboptimal and there is mixed success in diluting into liquid media with insufficient CFU·mL⁻¹ concentration. The measure is growth over starting inoculum, but we are not told if there was a control well with no subtilosin addition, which would have been the appropriate comparison [53]. Presented here regardless of capsule formation, we observed inhibition of *P. gingivalis* growth; however, we did observe that those strains without or with little capsule, 381 and 33,277 respectively, were slightly more susceptible to be killed by the extract, which was in agreement with the previous finding [53].
Interestingly, Lys-gingipains of *P. gingivalis* do not inhibit activity of subtilisin as reported by Shelburne *et al.* [53]. However, the studied *P. gingivalis* strain 33,277 was already remarkably sensitive to subtilisin. It will be interesting to elucidate susceptibility of *P. gingivalis* W83 to subtilisin since this strain is lacking some proteases and is likely to be more sensitive to the antimicrobial protein. The presence of the protease on the surface is likely to degrade subtilisin. However, it was shown that the mutant lacking the proteolytic enzyme coding gene displayed similar MIC to the parent strain. Arg-gingipain absence failed to induce further sensitivity to subtilisin. The authors state that this could be due to the lack of arginine residues in proteins. Subtilisin contains lysine residues, however, it is not noted if the correct gingipain cleavage site is present in subtilisin, therefore the lys-gingipain also has the potential to be ineffective at cleavage. It should be noted that *P. gingivalis* has a host of proteases, and given the robust inhibition of growth, it would appear that the agent responsible for activity is not susceptible to proteolysis by *P. gingivalis* proteases. To confirm, this hypothesis would need to be formally tested.

Previous studies by Tsubura *et al.* [54] have demonstrated that Extraction 300E (E-300, AHC Co., Gunma, Japan), a preparation from the culture medium supernatant of Japanese soil *B. subtilis* isolate and the commercially available VITALREX (AHC Co., Gunma, Japan), a stable oral tablet of lyophilized *B. subtilis* DB9011, were both effective in reducing periodontitis levels in patients in comparison to controls [54] [55]. The BANA tests that were used to assay for periodontitis showed reduced levels in patients treated with the compounds; this test indicates that there are organisms that are able to hydrolyze the synthetic peptide benzoyl-DL-arginine-naphthylamide, which are attributed to the Gram negative anaerobes present in the subgingival plaque, of which *P. gingivalis* is a member. A reduction in BANA levels also coincided with a reduction in a number of target bacteria, including *P. gingivalis* and *P. intermedia* [55].

It is being debated which tests may serve as a “minimum requirement” to characterize a putative probiotic’s safety and value [1] [56] [57]. Most agree that the antibiotic susceptibility of bacterial cells is the test of priority to identify if they are tolerant or sensitive to commonly prescribed antibiotics. Resistance of probiotics to antibiotics has both positive and negative impacts on human health. When bacterial resistance is intrinsic, it helps and supports the restoration of intestinal microbiota after a course of antibiotics that are administrated to the host for infection treatment [57]. At the same time, it is problematic when antibiotic resistance-genes in probiotics are transferable to other microbiota or pathogens leading to the appearance of new resistant strains [58]. In our study, we evaluated the antibiotic susceptibility, using a disc diffusion test, of the two *Bacillus* strains to nine antibiotics. The tested bacilli were more tolerant to bacitracin and streptomycin but susceptible to penicillin, ampicillin, erythromycin and chloramphenicol more than other used antibiotics. Also, tolerance of *Bacillus* was not a surprising finding and it was confirmed by genome annotation data that was performed by Karlyshev *et al.* [64] for the studied bacilli using RAST analysis [49]. In addition to multidrug resistance efflux pumps encoding genes in *B. subtilis* KATMIRA1933, genes coding for resistance to vancomycin, fluoroquinolones, fosfomycin, and β-lactam antibiotics were also identified. Importantly, these studies referred to the fact that the probiotic bacilli that are used in animal and human food industries have shown multi-drug resistance behavior, especially toward streptomycin and tetracycline [65]-[67]. In the case of *B. amyloliquefaciens* B-1895, the β-lactamase gene, the streptothricin acetyltransferase-biosynthesis gene, and genes for resistance to fluoroquinolone and tetracycline antibiotics were also detected [50].

Antibiotic resistance of bacterial species is either intrinsic or acquired by the transfer of a gene from plasmids, transposons, or the mutation of the bacterial gene [68] [69]. Mazza *et al.* [70] tested the resistance stability of antibiotic resistance markers existing in *B. subtilis* O/C, T, N/R, and SIN strains. Four therapeutic antibiotics were considered (chloramphenicol, tetracycline, rifampicin and streptomycin). They noticed that the resistance stability to tetracycline, rifampicin, and streptomycin existed for at least 200 generations without selective pressure. *In vivo* and *in vitro* studies explained the “absence of homologous transfer of resistance markers among the
resistant strains” [70]. Bacillus species such as B. subtilis have been included on the Qualified Presumption of Safety (QPS) microbes list. The QPS list was generated including microbial taxonomic units in which the acquired antibiotic resistance-genes are absent [3] [71].

In addition to antibiotic resistance, toxigenic potential such as the hemolytic activity [72] of Bacillus was evaluated in this study. It is known that hemolysin enzyme production is one of the virulence factors of pathogenic microorganisms. In whole blood agar, a weak hemolytic zone around B. subtilis KATMIRA1933, but not B. amyloliquefaciens B-1895, was observed and compared with the clearer zone of hemolysis produced by S. aureus, a positive control. No hemolytic activity of both B. subtilis KATMIRA1933 and B. amyloliquefaciens B-1895 was detected on defibrinated blood agar. Therefore, we assumed that the activity of B. subtilis KATMIRA1933 on the whole blood agar was fibrinolytic, not hemolytic action. To confirm our assumption, a fibrinolytic assay was performed for both tested bacilli. The clear zone of fibrinolytic activity produced by B. subtilis KATMIRA1933 was wider than the zone generated by the Bacillus strain B. amyloliquefaciens B-1895. The question that remains is why B. amyloliquefaciens B-1895, which has fibrinolytic activity like B. subtilis KATMIRA1933, did not produce the zone of hemolysis on the whole blood agar. However, hemolytic activity was determined in commercial human Bacillus species commonly used as probiotics [72] [73]. Hemolytic activity is a highly recommended test by EFSA to ensure that the bacterial strain was free of the toxigenic potential [74]. Although studies on fish and pigs reported that the hemolytic activity of microbial enzymes in vitro does not necessarily produce any negative effect in vivo [75] [76], hemolytically active bacteria are not recommended as feed additives according to EFSA guidelines [71]. Based on our results, we propose the evaluation of bacterial hemolytic activity accompanied by the assessment of fibrinolytic activity.

A bacterial reverse mutation (Ames) assay was performed to evaluate the mutagenic potential and ensure safe utilization of Bacillus’ metabolites. The mutant frequency value confirmed that CFSs of B. subtilis KATMIRA1933 and B. amyloliquefaciens B-1895 were free from mutagenic substances. In this work, we used S. typhimurium K-6 TA1535 as his-mutant strain without using a metabolic activator (S9 mix). Mortelmans and Zeiger [30], Vijayan et al. [77], and Lupi et al. [78] found no significant differences in the number of revertant colonies of S. typhimurium TA1535 in the presence or absence of S9. The results of the Ames test were considered as primary data but did not guarantee that bacilli cell products do not have any mutagenic or carcinogenic active substances [79]. We agree that some supportive tests are needed to strengthen our finding, such as the Micronucleus assay, in vitro chromosomal aberration assay, and oral toxicity studies in rats to identify genotoxicity and clastogenicity [79].

According to the WHO definition that probiotics produce health benefits in the host, proteolytic activity of the tested bacilli was evaluated and identified. Our data showed that B. subtilis KATMIRA1933 and B. amyloliquefaciens B-1895 behaved like many previously studied Bacillus species, showing a clear zone of proteolytic activity when they were inoculated on milk agar medium. Sevinc and Demirkan [80] found that utilizing skim milk agar for the qualitative test of protease is better than casein agar. The advantages of proteolytic enzymes have been reported in many studies [27]-[29]. In these studies, the importance of proteolytic enzymes includes the activation of regeneration processes, the enhancement of fibrinolytic activity in the plasma, the enhancement of normal digestion processes, and degradation of allergic and chemical compounds.

The ability of probiotics to co-aggregate and auto-aggregate is considered an advantageous characteristic feature. Their adhesion to a pathogenic organism can facilitate the elimination of the organism from the body and its ability to self-aggregate gives it an advantage in a competitive environment. The microorganisms employed in this study were chosen as common pathogenic organisms found in products of consumption. Two mathematical analyses of the coaggregation data were evaluated, Method 1 was described by Ledder et al. [35] and Method 2 was described by Handley et al. [45]. The more comprehensive method for mathematical interpretation of optical density data was chosen on the basis of two requirements. Foremost, the calculated percentage of coaggregation had to make sense in its application. A mathematical form of analysis may often fall short when the parameters of a biological system must be taken into account, not quite “fitting” in a representative attempt. Second of all, the percentages that better reflected the visual analysis were identified. The extent of coaggregation and auto-aggregation was determined from the data that was found using the more appropriate method.

Method 1 appeared to be most appropriate for the data analysis in this study, for it agreed with the microscopic (visual) observations (Figure 1). Method 2 failed to adequately reflect the adhesion in a number of mixtures of microorganisms that were easily observed microscopically. For instance, some Method 2 derived values
were <0, although coaggregation was clearly visible under the microscope (Figure 1). This method error could be due to any number of factors that affected the microorganisms, if the equation reflects a specific state. Method 1, however, gave percentages of coaggregation that more closely resembled the scores given during visual analysis, signifying that it was more appropriate for the data at hand.

After 2 h, B. amyloliquefaciens B-1895 adhered most to P. aeruginosa and E. coli more than other bacterial cells. Though these data did ideally match the coaggregation percentages of B. amyloliquefaciens B-1895 with P. aeruginosa and E. coli, which were found to have 31.5% and 31.4%, respectively, at this optical density when method 1 was applied (Table 6).

B. subtilis KATMIRA1933 adhered mostly to E. coli and P. aeruginosa, but less to S. enterica, S. aureus, and L. monocytogenes (Table 7). Similarly, B. subtilis KATMIRA1933 mostly coaggregated with the above-mentioned bacterial cells. Both Bacillus strains were found to have auto-aggregating abilities after 2 h, especially B. amyloliquefaciens B-1895 (86%), which was also reflected in the visual analysis (data not shown).

According to the percentages of auto-aggregation (Table 7), B. amyloliquefaciens B-1895 showed greater instances of auto-aggregation than B. subtilis KATMIRA1933, at a more significant level, during 8 h. For B. amyloliquefaciens B-1895, the highest percentage of coaggregation was noticed with E. coli and P. aeruginosa and the lowest was with S. aureus (Table 8). B. subtilis KATMIRA1933 was highly coaggregated with E. coli, P. aeruginosa, and L. monocytogenes but less with S. mutans (Table 8).

Spores of B. subtilis KATMIRA1933 and B. amyloliquefaciens B-1895 showed high tolerance to different pH values of 2.0, 2.5, and 3.0 that simulate gastric conditions. The survival rates (%) of SFU in various acidic conditions during 4 h of incubation were either similar or less than 1-log reduction of the viable spore count when compared with a control (at time zero), and more than 80% of Bacillus spores survived in 0.3% bile salts (Data not shown). Duc et al. [81] stated that not all the spores of Bacillus probiotic strains were tolerant to gastric acidity and bile compounds in the upper gastrointestinal tract. Diversity in acid and bile tolerance of Bacillus species spores has been detected. When B. coagulans Unique IS-2 spores were evaluated by Sudha et al. [82], a 2-log reduction of SFU mL⁻¹ was identified after exposure to pH 1.5 and a 1-log reduction at both pH 2.0 and 3.0 at 3 h. Also, three strains of B. coagulans (BC14 LMAB, CIP5264 and CIP6625) were tested by Hyronimus et al. [47], who reported the high susceptibility of these strains to acid (pH 2.5) and 0.3% bile after 3 h incubation. Our findings were in agreement with Guo et al. [83] who found that the number of B. subtilis MA139 that exerted probiotic potential in the gastrointestinal tract of pigs, was steady (unaffected) at pH 2.0 and in nutrient broth supplemented with 0.3% bile for 3 h. The high tolerance of Bacillus spores to gastric acidity and bile salts of the proximal intestine, compared with vegetative cells, are asorted properties required for the selection of probiotics [84] and are promising in acidified food packaging and oral pharmaceutical applications.

Germination of Bacillus spores in the intestinal environment has been mentioned in many studies [73] [85] [86]. Studies such as Barbosa et al. [87], Duc et al. [81] and Fakhry et al. [88] were conducted to evaluate the tolerance of vegetative cells of Bacillus to acids and bile salts. Our data were in agreement with these studies in which high susceptibility of cells, in contrast to spores, to both conditions was reported. Studies of spore structures are required to determine the correlation with the susceptibility of some Bacillus spores to acids or bile salts. Evaluation of the survivability of spores in the presence of gastrointestinal tract enzymes, lysozymes, acids and bile salts [84] [86] need to be conducted in vivo to reflect the real assessment of Bacillus stability (sporulation or germination) under such conditions.

Before the establishment of probiotic capacity, other properties of B. subtilis KATMIRA1933 and B. amyloliquefaciens B-1895 should be further evaluated such as cell adhesion, hydrophobicity, and genotoxicity. To conclude, safety of the studied bacilli to human health should be established and completely confirmed due to the exciting potential of Bacillus for personal care, food and medical applications.

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Conflict of Interest

The authors declare no conflict of interest.
References

[1] FAO/WHO (2006) Probiotics in Food. Health and Nutritional Properties and Guidelines for Evaluation. FAO Food and Nutritional, Paper No. 85.

[2] Hong, H.A., Duc, L.H. and Cutting, S.M. (2005) The Use of Bacterial Spore Formers as Probiotics. FEMS Microbiology Review, 29, 813-835. http://dx.doi.org/10.1016/j.femsre.2004.12.001

[3] Scientific Committee of the European Food Safety Authority (2007) Introduction of a Qualified Presumption of Safety (QPS) Approach for Assessment of Selected Microorganisms Referred to EFSA—Opinion of the Scientific Committee. The EFSA Journal, 587, 1-16.

[4] Leyer, G.J., Li, S., Mubasher, M., Reifer, C. and Ouweland, A.C. (2009) Probiotic Effects on Cold and Influenza-Like Symptom Incidence and Duration in Children. Pediatrics, 124, 172-179. http://dx.doi.org/10.1542/peds.2008-2666

[5] Yamashiro, Y. and Nagata, S. (2010) Beneficial Microbes for Premature Infants, and Children with Malignancy Undergoing Chemotherapy. Beneficial Microbes, 1, 357-365. http://dx.doi.org/10.3920/BM2010.0035

[6] Thirabunyanon, M. and Thongwittaya, N. (2012) Protection Activity of a Novel Probiotic Strain of Bacillus subtilis against Salmonella Enteritidis Infection. Research in Veterinary Science, 93, 74-81. http://dx.doi.org/10.1016/j.rvsc.2011.08.008

[7] Allen, H.K., Trachsel, J., Looff, T. and Casey, T.A. (2014) Finding Alternatives to Antibiotics. Annals of the New York Academy of Sciences, 1323, 91-100. http://dx.doi.org/10.1111/nyas.12468

[8] Khochamit, N., Siripornadulsil, S., Sukon, P. and Siripornadulsil, W. (2015) Antibacterial Activity and Genotypic-Phenotypic Characteristics of Bacteriocin-Producing Bacillus subtilis K1213: Potential as a Probiotic Strain. Microbiological Research, 170, 36-50. http://dx.doi.org/10.1016/j.micres.2014.09.004

[9] Sutyak, K.E., Wirawan, R.E., Aroucheva, A.A. and Chikindas, M.L. (2008) Isolation of the Bacillus subtilis Antimicrobial Peptide Subtilisin from the Dairy Product-Derived Bacillus amyloliquefaciens. Journal of Applied Microbiology, 104, 1067-1074. http://dx.doi.org/10.1111/j.1365-2672.2007.03626.x

[10] Gandhi, M. and Chikindas, M.L. (2007) Listeria: A Foodborne Pathogen That Knows How to Survive. International Journal Food Microbiology, 113, 1-15. http://dx.doi.org/10.1016/j.ijfoodmicro.2006.07.008

[11] Golovko, G.V., Zipelt, L.I., Karpenko, G.I., Chistyakov, V.A., Sazykina, M.A. and Kolenko, M.A. (2008) Method for Growth of Young Azov-Chernomorskaya Royal Fish in Ponds. RU Patent No. 2376755.

[12] Molnár, A.K., Podmaniczky, B., Kúrti, P., Tenk, I., Glávits, R., Virág, G. and Szabó, Z. (2011) Effect of Different Concentrations of Bacillus subtilis on Growth Performance, Carcase Quality, Gut Microflora and Immune Response of Broiler Chicks. British Poultry Science, 52, 658-665. http://dx.doi.org/10.1080/00071668.2011.636029

[13] Chistyakov, V., Melnikov, V., Chikindas, M.L., Khutishvili, M., Chagelishvili, A., Bren, A., Kostina, N., Caver, V. and Eliashvili, V. (2015) Poultry-Beneficial Solid-State Bacillus amyloliquefaciens B-1895 Fermented Soybean Formula. Bioresource and Biotechnology, 3, 25-28. http://dx.doi.org/10.12938/bmfh.2014-012

[14] Przadnlowa, E.V., Chistyakov, V.A., Churilov, M.N., Mazanko, M.S., Bren, A.B., Volski, A. and Chikindas, M.L. (2015) DNA-Protection and Antioxidant Properties of Fermentates from Bacillus amyloliquefaciens B-1895 and Bacillus subtilis KATMIRA933. Letters in Applied Microbiology, 61, 549-554. http://dx.doi.org/10.1111/lam.12491

[15] Inglis, V. (1999) Antibacterial Chemotherapy and Drug Resistance in Aquaculture. In: Karunasagar, I., Karunasagar, I. (Eds.), Aquaculture and Biotechnology, Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi, 135-155.

[16] Cartwright, P. (2009) Bacillus subtilis—Identification and Safety. Probiotics News, No. 2, 1-3.

[17] Monod, M., Denoya, C. and Dubna, D. (1986) Sequence and Properties of pMI13, Amacrolide-Lincosamide-Streptogramin B Resistance Plasmid from Bacillus subtilis. Journal of Bacteriology, 167, 138-147.

[18] Roberts, A.P., Pratten, J., Wilson, M. and Mullany, P. (1999) Transfer of a Conjugative Transposon, Tn5397 in a Mod- el Oral Biofilm. FEMS Microbiology Letters, 177, 63-66. http://dx.doi.org/10.1016/S0168-6445(99)00252-0

[19] DAI, L., WU, C.M., WANG, M.G., WANG, Y., HUANG, S.Y., XIA, L.N., LI, B.B. and SHEN, J.Z. (2010) First Report of the Multi-Drug Resistance Gene fexA and the Phenicol Resistance Gene fexA of Bacillus subtilis Strain from Swine Feces. Antimicrobial Agents and Chemotherapy, 54, 3953-3955. http://dx.doi.org/10.1128/AAC.00169-10

[20] Phelan, R.W., O’Halloran, A., Kennedy, J., Morrissey, J.P., Dobson, A.D., O’Gara, F. and Barbosa, T.M. (2011) Tetraacycline Resistance-Encoding Plasmid from Bacillus sp. Strain #24, Isolated from the Marine Sponge Haliclonia simpulins. Applied and Environmental Microbiology, 77, 327-329. http://dx.doi.org/10.1128/AEM.01239-10

[21] Rosendal, S., Devenish, J., MacInnes, J.I., Lumsden, J.H., Watson, S. and Xun, H. (1988) Evaluation of Heat-Sensitive, Neutrophil-Toxic, and Hemolytic Activity of Haemophilus (Actinobacillus) pleuroneumoniae. American Journal of Veterinary Research, 49, 1053-1058.

[22] Arzanlou, M. and Bohlooli, S. (2010) Inhibition of Streptolysin O by Allicin—An Active Component of Garlic. Jour-
[23] Luo, C., Liu, X., Zhou, H., Wang, X. and Chen, Z. (2014) Identification of Four NRPS Gene Clusters in Bacillus subtilis 916 for Four Families of Lipopeptides Biosynthesis and Evaluation of Their Intricate Functions to the Typical Phenotypic Features. Applied and Environmental Microbiology, 81, 422-443. http://dx.doi.org/10.1128/AEM.02921-14

[24] Pan, X., Chen, X., Su, X., Feng, Y., Tao, Y. and Dong, Z. (2014) Involvement of SpoVG in Hemolysis Caused by Bacillus subtilis. Biochemical and Biophysical Research Communications, 443, 899-904. http://dx.doi.org/10.1016/j.bbrc.2013.12.069

[25] Goebel, W., Chakraborty, T. and Kreft, J. (1988) Bacterial Hemolysins as Virulence Factors. Anton van Leeuwenhoek, 54, 453-463. http://dx.doi.org/10.1007/BF00461864

[26] Liu, L., Liu, Y.F., Shin, H.D., Chen, R.R., Wang, N.S., Li, J., Du, G. and Chen, J. (2013) Developing Bacillus spp. as a Cell Factory for Production of Microbial Enzymes and Industrially Important Biochemicals in the Context of Systems and Synthetic Biology. Applied Microbiology and Biotechnology, 97, 6113-6127. http://dx.doi.org/10.1007/s00253-013-4960-4

[27] Gupta, R., Beg, Q.K. and Lorenz, P. (2002) Bacterial Alkaline Proteases: Molecular Approaches and Industrial Applications. Applied Microbiology and Biotechnology, 59, 15-32. http://dx.doi.org/10.1007/s00253-002-0975-y

[28] Rojas, J.B.U., Verreth, J.A.J., Amato, S. and Huisman, E.A. (2003) Biological Affects Treatment the Chemical Composition of Coffee Pulp. Bioresource Technology, 89, 267-274. http://dx.doi.org/10.1016/S0960-8524(03)00070-1

[29] Phromraksa, P., Nagano, H., Boomars, T. and Kamboonruang, C. (2008) Identification of Proteolytic Bacteria from Thai Traditional Fermented Foods and Their Allergenic Reducing Potentials. Journal of Food Science, 73, M189-M195. http://dx.doi.org/10.1111/j.1750-3841.2008.00721.x

[30] Mortelmans, K. and Zeiger, E. (2000) The Ames Salmonella/Microsome Mutagenicity Assays. Mutation Research/ Fundamental and Molecular Mechanisms of Mutagenesis, 455, 29-60. http://dx.doi.org/10.1016/S0022-2408(00)00064-6

[31] Loeb, K.R. and Loeb, L.A. (2000) Significance of Multiple Mutations in Cancer. Carcinogenesis, 21, 379-385. http://dx.doi.org/10.1093/carcin/21.3.379

[32] Succi, M., Tremonte, P., Reale, A., Sorrentino, E., Grazia, L., Pacifico, S. and Coppola, R. (2005) Bile Salt and Acid Tolerance of Lactobacillus rhamnosus Strains Isolated from Parmigiano Reggiano Cheese. FEMS Microbiology Letters, 244, 129-137. http://dx.doi.org/10.1016/j.femsle.2005.01.037

[33] Erkkilä, S. and Petäjä, E. (2000) Screening of Commercial Meat Starter Cultures at Low pH and in the Presence of Bile Salts for Potential Probiotic Use. Meat Science, 55, 297-300. http://dx.doi.org/10.1016/S0309-1740(99)00156-4

[34] Huang, Y. and Adams, M.C. (2004) In Vitro Assessment of the Upper Gastrointestinal Tolerance of Potential Probiotic Dairy Propionibacteria. International Journal of Food Microbiology, 91, 253-260. http://dx.doi.org/10.1016/j.ijfoodmicro.2003.07.001

[35] Ledder, R.G., Timperley, A.S., Friswell, M.K., Macfarlane, S. and McBain, A.J. (2008) Coaggregation between and Among Human Intestinal and Oral Bacteria. FEMS Microbiology Ecology, 66, 630-636. http://dx.doi.org/10.1111/j.1574-6941.2008.00525.x

[36] Kos, B., Suskovic, J., Vukovic, S., Simpraga, M., Frece, J. and Matosic, S. (2003) Adhesion and Aggregation Ability of Probiotic Strain Lactobacillus acidophilus M92. Journal of Applied Microbiology, 94, 981-987. http://dx.doi.org/10.1046/j.1365-2672.2003.01915.x

[37] Clinical and Laboratory Standards Institute (CLSI) (2012) Performance Standards for Antimicrobial Disk Susceptibility Tests. 11th Edition, Approved Standard, M02-A11.

[38] Maron, D. and Ames, B. (1983) Revised Methods for the Salmonella Mutagenicity Test. Mutation Research/Environmental Mutagenesis and Related Subjects, 113, 173-215. http://dx.doi.org/10.1016/0165-1161(83)90010-9

[39] Cappuccino, J.G. and Sherman, N. (2004) Microbiology: A Laboratory Manual. Pearson Education, Singapore, 491.

[40] Kirkland, D.J. (1989) Statistical Evaluation of Mutagenicity Test Data, UKEMS Subcommittee on Guidelines for Mutagenicity Testing. Cambridge University Press, Cambridge, 102-140.

[41] Ponmurugan, P. (2007) Proteolytic Activity of Bacillus cereus under in Vitro Condition. Journal of Biological Sciences, 7, 63-67. http://dx.doi.org/10.3923/jbs.2007.63.67

[42] Uyar, F., Porsuk, I., Kizil, G. and Yilmaz, E.I. (2011) Optimal Conditions for Production of Extracellular Protease from Newly Isolated Bacillus cereus Strain CA15. EurAsian Journal of BioSciences, 5, 1-9.

[43] Luo, G., Samaranayake, L.P. and Yau, J.Y. (2001) Candida Species Exhibit Differential in Vitro Hemolytic Activities. Journal of Clinical Microbiology, 39, 2971-2974. http://dx.doi.org/10.1128/JCM.39.8.2971-2974.2001

[44] Cisar, J.O., Kolenbrander, P.E. and McIntire, F.C. (1979) Specificity of Coaggregation Reactions between Human Oral
Streptococci and Strains of *Actinomyces viscosus* or *Actinomyces naeslundii*. *Infection and Immunity*, 24, 742-752.

[45] Handley, P.S., Harty, D.W., Wyatt, J.E., Brown, C.R., Doran, J.P. and Gibbs, A.C. (1987) A Comparison of the Adhesion, Coaggregation and Cell-Surface Hydrophobicity Properties of Fibrillar and Fimbriate Strains of *Streptococcus salivarius*. *Journal of General Microbiology*, 133, 3207-3217.

[46] Franklin, M.L. and Clark, W.A. (1981) Simple, Inexpensive, and Rapid Way to Produce *Bacillus subtilis* Spores for the Guthrie Bioassay. *Journal of Clinical Microbiology*, 14, 113-115.

[47] Hyronimus, B., Le, M.C., Hadj, S.A. and Deschamps, A. (2000) Acid and Bile Tolerance of Spore-Forming Lactic Acid Bacteria. *International Journal of Food Microbiology*, 61, 193-197. http://dx.doi.org/10.1016/S0168-1605(00)00366-4

[48] Clinical and Laboratory Standards Institute (CLSI) (2007) Performance Standards for Antimicrobial Susceptibility Testing. 17th Edition, Approved Standard, M100-S17.

[49] Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., Kubal, M., Meyer, F., Olsen, G.J., Olson, R., Osterman, A.L., Overbeek, R.A., McNeil, L.K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G.D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A. and Zagnitko, O. (2008) The RAST Server: Rapid Annotations Using Subsystems Technology. *BMC Genomics*, 9, 75. http://dx.doi.org/10.1186/1471-2164-9-75

[50] Karlyshev, A.V., Melnikov, V.G. and Chistyakov, V.A. (2014) Draft Genome Sequence of *Bacillus amyloliquefaciens* B-1895. *Genome Announcements*, 2, e00633-e00614. http://dx.doi.org/10.1128/genomeA.00633-14

[51] Turovskiy, Y., Cheryian, T., Algburi, A., Wirawan, R.E., Tkhivistov, P., Sinko, P.J. and Chikindas, M.L. (2012) Susceptibility of *Gardnerella vaginalis* Biofilms to Natural Antimicrobials Subtilosin, ε-Poly-L-Lysine, and Lauramide Arginine Ethyl Ester. *Infectious Diseases in Obstetrics and Gynecology*, 2012, Article ID: 284762. http://dx.doi.org/10.1155/2012/284762

[52] Algburi, A., Volski, A. and Chikindas, M.L. (2015) Natural Antimicrobials Subtilosin and Lauramide Arginine Ethyl Ester (LAE) Synergize with Conventional Antibiotics Clindamycin and Metronidazole against Biofilms of *Gardnerella vaginalis* but Not against Biofilms of Healthy Vaginal Lactobacilli. *Pathogens and Disease*, 73, ftv018.

[53] Shelburne, C.E., An, F.Y., Dholpe, V., Ramamoorthy, A., Lopatin, D.E. and Lantz, M.S. (2007) The Spectrum of Antimicrobial Activity of the Bacteriocin Subtilosin A. *Journal of Antimicrobial Chemotherapy*, 61, 193-197. http://dx.doi.org/10.1093/jac/dkl495

[54] Tsubura, S., Mizunuma, H., Ishikawa, S., Oyake, I., Okabayashi, M., Katoh, K., Shibata, M., lizuka, T., Toda, T. and Iizuka, T. (2009) The Effect of *Bacillus subtilis* Mouth Rinsing in Patients with Periodontitis. *European Journal of Clinical Microbiology and Infectious Diseases*, 28, 1353-1356. http://dx.doi.org/10.1007/s10096-009-0790-9

[55] Tsubura, S., Waki, Y. and Tsubura, T. (2012) Probiotic Effect of *Bacillus subtilis* Tablets on Periodontopathic Oral Bacteria. *Microbiology Research*, 3, 94-98.

[56] Ganguly, N.K., Bhattacharya, S.K., Sesikeran, B., Nair, G.B., Ramakrishna, B.S., Sachdev, H.P., Batish, V.K., Kanagasabapathy, A.S., Muthuswamy, V., Kathuria, S.C., Katoch, V.M., Satyanarayana, K., Toteja, G.S., Rahi, M., Rao, S., Bhan, M.K., Kapur, R. and Hemalatha, R. (2011) ICMR-DBT Guidelines for Evaluation of Probiotics in Food. *Indian Journal of Medical Research*, 134, 22-25.

[57] Gueimonde, M., Sánchez, B., De los Reyes-Gavilán, C.G. and Margolles, A. (2013) Antibiotic Resistance in Probiotic Bacteria. *Frontiers in Microbiology*, 4, 1-6. http://dx.doi.org/10.3389/fmicb.2013.00202

[58] Aquilanti, L., Garofalo, C., Osimani, A., Silvestri, G., Vignaroli, C. and Clementi, F. (2007) Isolation and Molecular Characterization of Antibiotic-Resistant Lactic Acid Bacteria from Poultry and Swine Meat Products. *Journal of Food Protection*, 70, 557-565.

[59] Podlesek, Z., Comino, A., Herzog-Velikonja, B., Zgur-Bertok, D., Komel, R. and Grabnar, M. (1995) *Bacillus licheniformis* Bacitracin-Resistance ABC Transporter: Relationship to Mammalian Multidrug Resistance. *Molecular Microbiology*, 16, 969-976. http://dx.doi.org/10.1111/j.1365-2958.1995.tb02322.x

[60] Cain, B.D., Norton, P.J., Eubanks, W., Nick, H.S. and Allen, C.M. (1993) Amplification of the *bca* Gene Confers Bacitracin Resistance to *Escherichia coli*. *Journal of Bacteriology*, 175, 3784-3789.

[61] Cao, M. and Helmann, J.D. (2002) Regulation of the *Bacillus subtilis* bcrC Gene by Two Extracytoplasmic f Factors. *Journal of Bacteriology*, 184, 6123-6129. http://dx.doi.org/10.1128/JB.184.22.6123-6129.2002

[62] Senesi, S., Celandroni, F., Tavanti, A. and Ghelardi, E. (2001) Molecular Characterization and Identification of *Bacillus clausii* Strains Marketed for Use in Oral Bacteriotherapy. *Applied and Environmental Microbiology*, 67, 834-839. http://dx.doi.org/10.1128/AEM.67.2.834-839.2001

[63] Adimpong, D.B., Sørensen, K.I., Thorsen, L., Stuer-Lauridsen, B., Abdelgardir, W.S., Nielsen, D.S., Derkx, P.M.F. and Jespersen, L. (2012) Antimicrobial Susceptibility of *Bacillus* Strains Isolated from Primary Starters for African Tradi-
tional Bread Production and Characterization of the Bacitracin Operon and Bacitracin Biosynthesis. Applied and Environmental Microbiology, 78, 7903-7914. http://dx.doi.org/10.1128/AEM.00730-12

[64] Karlyshev, A.V., Melnikov, V.G. and Chikindas, M.L. (2014) Draft Genome Sequence of Bacillus subtilis Strain KATMIRA1933. Genome Announcements, 2, e00619-e00614. http://dx.doi.org/10.1128/genomeA.00619-14

[65] Ciffo, F. (1984) Determination of the Spectrum of Antibiotic Resistance of the “Bacillus subtilis” Strains of Enterococci. Chemioterapia, 3, 45-52.

[66] Hoa, N.T., Baccigalupi, L., Huxham, A., Smertenko, A., Van, P.H. and Ammendola, S. (2000) Characterization of Bacillus Species Used for Oral Bacteriotherapy and Bacterioprophylaxis of Gastrointestinal Disorders. Applied and Environmental Microbiology, 66, 5241-5247. http://dx.doi.org/10.1128/AEM.66.12.5241-5247.2000

[67] Green, D.H., Wakeley, P.R., Page, A., Barnes, A., Baccigalupi, L., Ricca, E. and Cutting, S.M. (1999) Characterization of Two Bacillus Probiotics. Applied Environmental Microbiology, 65, 4288-4291.

[68] Health and Consumer Protection Directorate—General of the European Commission (HCPDGEC) (2002) Opinion of the Scientific Committee on Animal Nutrition on the Criteria for Assessing the Safety of Microorganism Resistant to Antibiotics of Human Clinical and Veterinary Importance. Brussels, Belgium: Health and Consumer Protection Directorate. European Commission, 1-10.

[69] European Food Safety Authority (2012) Scientific Opinion on Guidance on the Assessment of Bacterial Susceptibility to Antimicrobials of Human and Veterinary Importance. EFSA Journal, 10, 2740-2750.

[70] Mazza, P., Zani, F. and Martelli, P. (1992) Studies on the Antibiotic Resistance of Bacillus subtilis Strains Used in Oral Bacteriotherapy. Bollettino Chimico Farmaceutico, 131, 401-408.

[71] European Food Safety Authority (2011) Scientific Opinion on the Maintenance of the List of QPS Biological Agents Intentionally Added to Food and Feed. EFSA Journal, 9, 2497.

[72] Duc, L.H., Hong, H.A. and Cutting, S.M. (2003) Germination of the Spore in the Gastrointestinal Tract Provides a Novel Route for Heterologous Antigen Delivery. Vaccine, 21, 4215-4224. http://dx.doi.org/10.1016/S0264-410X(03)00492-4

[73] Hoa, T.T., Duc, L.H., Isticato, R., Baccigalupi, L., Ricca, E. and Van, P.H. (2001) Fate and Dissemination of Bacillus subtilis Spores in a Murine Model. Applied Environmental Microbiology, 67, 3819-3823. http://dx.doi.org/10.1128/AEM.67.9.3819-3819.2001

[74] European Food Safety Authority (2011) Technical Guidance on the Assessment of the Toxigenic Potential of Bacillus Species Used in Animal Nutrition. EFSA Journal, 9, 2445.

[75] Patel, A.K., Deshhattiwar, M.K., Chaudhari, B.L. and Chincholkar, S.B. (2009) Production, Purification and Chemical Characterization of the Catecholate Siderophore From Potent Probiotic Strains of Bacillus spp. Bioresource Technology, 100, 368-373. http://dx.doi.org/10.1016/j.biotech.2008.05.008

[76] Trapecar, M., Leouffre, T., Faure, M., Jensen, H.E., Granum, P.E., Cencic, A. and Hardy, S.P. (2011) The Use of a Porcine Intestinal Cell Model System for Evaluating the Food Safety Risk of Bacillus cereus Probiotics and the Implications for Assessing Enterotoxicigenicity. APMIS, 119, 877-884. http://dx.doi.org/10.1111/j.1600-0463.2011.02797.x

[77] Vijayan, M., Deecaraman, M. and Pudupalayam, K.T. (2007) In Vitro Genotoxicity of Piperacillin Impurity-A. African Journal of Biotechnology, 6, 2074-2077.

[78] Lupi, S., Marconi, S., Paiaro, E., Fochesato, A. and Gregorio, P. (2009) Mutagenicity Evaluation with Ames Test of Hydro-Alcoholic Solution of Terpenes. Journal of Preventive Medicine and Hygiene, 50, 170-174.

[79] Endres, J.R., Clewell, A., Jade, K.A., Farber, T., Hauswirth, J. and Schauss, A.G. (2009) Safety Assessment of a Proprietary Preparation of a Novel Probiotic, Bacillus coagulans, as a Food Ingredient. Food and Chemical Toxicology, 47, 1231-1238. http://dx.doi.org/10.1016/j.fct.2009.02.018

[80] Sevinc, N. and Demirkan, E. (2011) Production of Protease by Bacillus sp. N-40 Isolated from Soil and Its Enzymatic Properties. Journal of Biological and Environmental Sciences, 5, 95-103.

[81] Duc, L.H., Hong, H.A., Barbosa, T.M., Henriques, A.O. and Cutting, S.M. (2004) Characterization of Bacillus Probiotics Available for Human Use. Applied and Environmental Microbiology, 70, 2161-2171. http://dx.doi.org/10.1128/AEM.70.4.2161-2171.2004

[82] Sudha, R., Chauhan, P. and Dixit, K. (2010) Molecular Typing and Probiotic Attributes of a New Strain of Bacillus coagulans—Unique IS-2: A Potential Biotherapeutic Agent. Genetic Engineering and Biotechnology Journal, 2010, 1-20.

[83] Guo, X., Li, D., Lu, W., Piao, X. and Chen, X. (2006) Screening of Bacillus Strains as Potential Probiotics and Subsequent Confirmation of the in Vivo Effectiveness of Bacillus subtilis MA139 in Pigs. Antonie Van Leeuwenhoek, 90, 139-146. http://dx.doi.org/10.1007/s10482-006-9067-9

[84] Salminen, S., Isolauri, E. and Salminen, E. (1998) Clinical Uses of Probiotics for Stabilizing the Gut Mucosal Barrier:
coli and Tannerella forsythia, the Red Bacterial Complex Associated with Periodontitis. Pathologic Biologie, 55, 154-162.  
http://dx.doi.org/10.1016/j.patbio.2006.07.045

[107] Lamont, R.J. and Jenkinson, H.F. (1998) Life below the Gum Line: Pathogenic Mechanisms of Porphyromonas gingivalis. Microbiology and Molecular Biology Reviews, 62, 1244-1263.

[108] Marchesan, J.T., Gerow, E.A., Schaff, R., Taut, A.D., Shin, S.Y., Sugai, J., Brand, D., Burberry, A., Jorns, J., Lundy, S.K., Nuñez, G., Fox, D.A. and Giannobile, W.V. (2013) Porphyromonas gingivalis Oral Infection Exacerbates the Development and Severity of Collagen-Induced Arthritis. Arthritis Research and Therapy, 15, R186.  
http://dx.doi.org/10.1186/ar4376

[109] Socransky, S.S. and Haffajee, A.D. (1992) Current Concepts of Bacterial Etiology. Journal of Periodontology, 36, 322-331.  
http://dx.doi.org/10.1902/jop.1992.63.4s.322

[110] Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M. and Tauxe, R.V. (1999) Food-Related Illness and Death in the United States. Emerging Infectious Diseases, 5, 607-625.  
http://dx.doi.org/10.3201/eid0505.990502

[111] Moore, W.E. and Moore, L.V. (1994) The Bacteria of Periodontal Diseases. Periodontology, 5, 66-77.  
http://dx.doi.org/10.1111/j.1600-0757.1994.tb00019.x

[112] Strauss, J., Kaplan, G.G., Beck, P.L., Rioux, K., Panaccione, R., Devinney, R., Lynch, T. and Allen-Vercoe, E. (2000) Invasive Potential of Gut Mucosa-Derived Fusobacterium nucleatum Positively Correlates with IBD Status of the Host. Inflammatory Bowel Diseases, 17, 1971-1978.  
http://dx.doi.org/10.1002/ibd.21606

[113] Han, Y.W., Shen, T., Chung, P.I., Buhimschi, A. and Buhimschi, C.S. (2009) Uncultivated Bacteria as Etiologic Agents of Intra-Amniotic Inflammation Leading to Preterm Birth. Journal of Clinical Microbiology, 47, 38-47.  
http://dx.doi.org/10.1128/JCM.01206-08

[114] Castellarin, M., Warren, R.L., Freeman, J.D., Dreolini, L., Krzywinski, M., Strauss, J., Barnes, R., Watson, P., Allen-Vercoe, E., Moore, R.A. and Holt, R.A. (2012) Fusobacterium nucleatum Infection Is Prevalent in Human Colorectal Carcinoma. Genome Research, 22, 299-306.  
http://dx.doi.org/10.1101/gr.126516.111