Assessment of temperature and acid tolerance of *Bacillus subtilis* isolated from a Brazilian fruit juice-added soy beverage

Avaliação da tolerância à temperatura e à acidez de Bacillus subtilis isolado de uma bebida de soja com suco de frutas brasileiro

Renato Ventresqui Oliveira[1]
Lívio da Silva Amaral[2]
Cristiane Aparecida Milagres[3]
Celso Tadeu Barbosa Santos[1]
Afonso Pelli[1]
Aline Dias Paiva[1]

[1] Universidade Federal do Triângulo Mineiro, Instituto de Ciências Biológicas e Naturais. E-mail: ventresqui@gmail.com*
[2] Instituto Federal de Educação, Ciência e Tecnologia Baiano, Campus Xique- Xique.
[3] MSM Indústria e Comércio Ltda

Abstract: Bacillus subtilis is a spore-forming bacterium and an important food contaminant. The aim of this study was to analyze the ability of B. subtilis spores to survive under conditions of low pH and high temperature. The package was purchased at a local supermarket, in Uberaba, Minas Gerais. A sample was collected, diluted and plated on Brain-Heart-Infusion agar (BHI). After incubation, suspected colonies of B. subtilis were transferred to BHI agar. Cell morphology, the presence of spores and Gram stain were examined, and the isolate was identified by 16S rRNA gene sequencing . The microscope evaluation indicated the presence of spores. The thermal tolerance of the spores was evaluated by the addition of $3x10^9$spores/mL in test tubes containing peptone water. Heat treatments were carried out at 80 and 90°C at different incubation times (0, 10, 20, 30, 40, 50 and 60 min). After heating, the tubes were cooled and the number of viable spores was determined in BHI Agar. For the analysis of spore survival, D and Z values were calculated. Tolerance to acid conditions was evaluated using BHI broth with different pH values. After incubation, the bacterial concentration was determined by determining viable cell count on BHI Agar medium. The vegetative cells were

transferred to the BHI broth and the pH was adjusted to different values (3, 4 or 5). Sampling were taken 8, 12 and 24 h after incubation. The samples were serially diluted in peptone water and spread in BHI Agar to determine the viable cell count . The 16S rRNA gene sequencing indicated high similarity (99.99%) with B. subtilis. D values were 17.01 min at 80°C and 13.42 min at 90°C. The Z-value was 97.13°C. B. subtilis was not able to grow at pH 3 and pH 4, but its survival was confirmed after the growth of colonies on BHI agar. At pH 5, B. subtilis grew after 24 h and the final pH changed to 7. Our results suggest that the spores of B. subtilis isolated from fruit juice-added soy beverage are tolerant to low pH and high temperature.

Keywords: Spores; Resistance; Contaminant; D- value; Z- value.

Resumo*: Bacillus subtilis é uma bactéria formadora de endósporos e um importante contaminante de alimentos. O objetivo deste estudo foi analisar a viabilidade dos endósporos de B.subtilis em condições de baixo pH e alta temperatura. A embalagem foi adquirida em um supermercado local, em Uberaba - Minas Gerais. A amostra foi coletada, diluída e plaqueada em meio Brain-Heart-Infusion (BHI). Após incubação, colônias suspeitas de B. subtilis foram transferidas para placas contendo meio BHI. A morfologia celular, a presença de endósporos e a coloração de Gram foram examinadas e o isolado foi identificado por sequenciamento do gene 16S rRNA. A tolerância térmica dos endósporos foi avaliada pela adição de $3x10^9$ endósporos/mL em tubos de ensaio contendo água peptonada. Os tratamentos térmicos foram realizados a 80 e 90 °C, em diferentes tempos de incubação (0, 10, 20, 30, 40, 50 e 60 min). Após aquecimento, os tubos foram resfriados e o número de endósporos viáveis foi determinado por contagem em placas em ágar BHI. Para a análise da sobrevivência dos endósporos foram calculados os valores D e o valor Z. A tolerância a condições ácidas foi avaliada em meio BHI líquido, com diferentes valores de pH. Após incubação foi determinada pela contagem de células viáveis, utilizando meio BHI. As células vegetativas foram transferidas para meio BHI líquido e o pH foi ajustado para diferentes valores (3, 4 ou 5). As coletas foram feitas 8, 12 e 24 h após a incubação. As amostras foram diluídas em água peptonada e plaqueadas em meio BHI para determinação de células viáveis (UFC/mL). O sequenciamento do gene 16S rRNA indicou alta similaridade (99,99%) com B. subtilis. Os valores D foram 17,01 min a 80 ° C e 13,42 min a 90 °C. O valor Z foi 97,13 °C. B. subtilis não foi capaz de crescer em pH 3 e pH 4, mas sua sobrevivência foi confirmada após o crescimento das colônias em ágar BHI. Em pH 5, B. subtilis apresentou crescimento após 24 h e o pH final foi alterado para 7. Os resultados sugerem que os endósporos de B. subtilis isolado de bebida de soja adicionada de suco de fruta são tolerantes a baixos valores de pH e altas temperaturas.*

Palavras-Chave: *Endósporos, Resistência, Contaminante, D- valor, Z- valor.*

INTRODUCTION

Bacillus subtilis is a Gram-positive, non-pathogenic, mesophilic, facultative anaerobic, spore-forming bacterium[1]. Spores are differentiated cellular structures produced by bacteria from the genera *Clostridium* and *Bacillus* in response to adverse environmental conditions and are considered a survival strategy[2].

Bacterial spores shown reduced metabolic activity and are highly resistant to extreme conditions that would normally be able to kill vegetative cells, including high temperatures, UV irradiation, desiccation and chemical damage. The resilience of *B. subtilis* spores makes them important food contaminants due to their strong resistance to microbial control strategies used in the food industry, such as pasteurization and most heating processes[2].

According to Karaman and Alvarez[3], bacteria that survive the heating process can cause deterioration of the milk, as is the case of *B. subtilis* where they observed a non-acidic curd that turned into a bitter taste. Moschonas et al.[4] observed that the presence of *B. subtilis* in vanilla cream pudding caused an increase in lactic acid concentration and in glucose and fructose concentrations, indicating loss of product quality. Logan[5] reports cases of illness caused by the consumption of food contaminated with *B. subtilis*, presenting vomiting and diarrhea as the most common symptoms, but points out that such cases are rare.

The aim of this study was to analyze the ability of *Bacillus subtilis* isolated from a Brazilian fruit juice-added soy beverage to survive under environmental stresses of low pH and high temperature.

METHODOLOGY

B. subtilis strain was isolated from an orange juice-added soy beverage purchased from a local supermarket, in Uberaba - Minas Gerais, Brazil. The package was stored at room temperature in a dry and well-ventilated room, as

recommended by the manufacturer. The package was stuffed, suggesting the presence of gas.

The whole package was sent to the laboratory and a sample was collected, under aseptic conditions, diluted, and plated on Brain-Heart-Infusion agar (BHI), using the pour plate method. After 24h of incubation at 37°C, bacterial colonies were harvested and sub-cultured on BHI agar[6].

Cell morphology and Gram staining were examined and the isolate was identified by 16S rRNA gene sequencing. Total DNA was extracted using the phenol-chloroform method[7]. The integrity of the extracted DNA was verified by agarose gel electrophoresis (1%) using 1X TBE buffer (90 mMTris base, 90 mM boric acid and 0.1 mM EDTA - pH 8.0). The gel was stained with ethidium bromide (0.2 µg/mL) and visualized using UV transilluminator. DNA concentration and purity were measured by Nano Drop (*Thermo Scientific*).

The 16S rRNA gene amplification reaction was performed in DNA thermal cycler Gene Amp PCR System 9700 (Applied Biosystems), using the pair of universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 536R (GTATTACCGCGGCTGCTG)[8]. The PCR reaction (25 µL) was composed by 17.8 µL of milli-Q water, 2.5 µL of 10X buffer, 0.625 µL of dNTP`s (10 mM), 1.25 µL of $MgCl_2$ (50 mM), 1 µL of primer F (10 pM/uL), 1 µL of primer R (10 pM/µL), 0.3 µL of Taq DNA polymerase (5 U/µL) and 0.6 µL of template DNA (50 ng/µL). The PCR conditions included an initial denaturation of 5 min at 95°C, followed by 35 cycles of 30 s at 95°C (denaturation), 30 s at 60°C (annealing) and 30 s at 72°C (polymerization). The amplification cycle was followed by a final extension of 7 min at 72°C, and the tubes were kept at 4°C. The PCR products were analyzed by agarose gel electrophoresis (1.5%) stained with ethidium bromide (0.2 µg/mL) and visualized using UV transilluminator.

The DNA sequencing was performed by the chain termination method using MegaBACE™ 1000 automated sequencer (GE Healthcare). All consensus sequences obtained were compared to those available in the GenBank database

(NCBI), and the alignment of the sequences was performed using the Basic Local Alignment Search Tool algorithm for nucleotide (BLASTn)[9].

The bacterium strain isolated was grown in *Bacillus cereus* agar, for 24h at 37°C. Stationary phase culture was diluted in peptone water (0.1 %) until obtaining an OD_{560} of 0.1. Examination of the culture in optical microscope indicated the presence of spores. Bacterial spores were obtained after heating the cell suspension at 80°C for 60 min[10]. The spore suspension was washed twice (15 min at 10000 rpm) in sterile saline solution (0.85% NaCl) and then resuspended in the same solution and enumerated by plating onto BHI agar. The result was expressed in colony forming units per milliliter (CFU/mL). The spore suspension was stored at 4°C until use.

The thermal tolerance of the bacterial spores was assayed by adding $3x10^9$ spores/mL into glass tubes containing peptone water (0.1%). Thermal treatments were carried out at 80 and 90°C at several incubation times (0, 10, 20, 30, 40, 50 and 60 min). After heating, the tubes were cooled in cooling bath (13ºC) and the viable spore number was determined by plate count in BHI agar after incubation at 37°C for 24 hours. The experiments were performed in duplicates.

For the analysis of bacterial spore survival, D-value was defined as the time in minutes at a given temperature necessary to decrease one $\log_{10}$ cycle of the bacterial spore number and Z-value was defined as the variation in temperature that reduced 10 times the D-value. D-values were calculated from the slope of the regression line obtained from the linear portion of the survival curve, which was in turn obtained from the plot of viable spore number ($\log_{10}$ values) versus heating time (minutes). Z-value was determined from the regression lines obtained by plotting $\log_{10}$ D-values versus the corresponding temperatures[11].

The tolerance to acidic conditions was evaluated using BHI broth with different pH values. Initially, the spore suspension was incubated in BHI broth, to allow the spore germination. After 24 hours at 37°C, the bacterial concentration was determined by viable count determination on BHI agar medium. The vegetative cells (10^3 CFU/mL) were transferred (1%) to BHI broth (20 mL) and the pH was

adjusted to different values (3, 4 or 5). Samplings were made at 8, 12 and 24 h after incubation at 37°C. The samples were serially diluted (10-fold) in peptone water and plated on BHI agar to determine the bacterial concentration (CFU/mL).

RESULTS

The bacterial colonies had morphological characteristics similar to *Bacillussp.*. Cells were rod-shaped and Gram-positive. The PCR reaction using the 16S universal primers 27F and 536R generated DNA fragments of approximately 500 bp. The amplicon was sequenced and compared to Gen Bank database using the BLASTn tool. High similarity (99,99%) was obtained with *Bacillus subtilis.*

After heat treatment, the time required to reduce one log cycle in viable *B. subtilis* spores was 17.01 min at 80°C and 13.42 min at 90°C (Figure 1). Z-value for *B. subtilis* spores in BHI medium was 97.13°C.

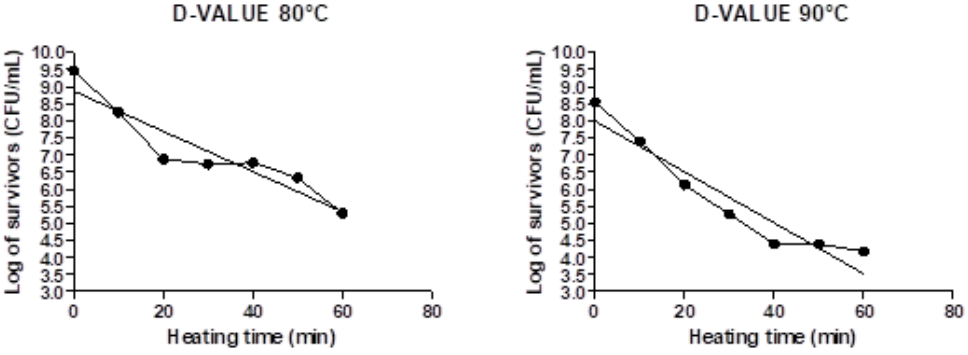

Figure 1. Thermal sensitivity of *B. subtilis* spores after heat treatment. D-values for 80 and 90° C.

Bacillus subtilis was not able to grow in BHI broth with pH 3 and pH 4 and no changes were observed in these treatments. The *B. subtilis* survival was confirmed after the growth of colonies on BHI agar. The concentration remained the same as at the time of the inoculation (XX CFU/ML). On the other hand, when cultured on initial pH 5, *B. subtilis* exhibited growth and final pH changed to 7 after 24 h of growing (Figure 2).

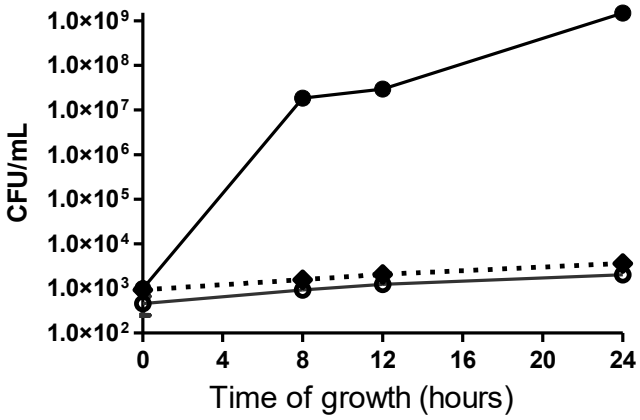

Figure 2. Effect of low pH on *B. subtilis* over 24 hours. Closed circles (●) represent the viability of *B. subtilis* in BHI broth on pH 5. Closed diamonds (♦) represent the viability of *B. subtilis* in BHI broth on pH 4. Opened circles (○) represent the viability of *B. subtilis* in BHI broth on pH 3.

DISCUSSION

In the process of food preservation, many stressful conditions for microorganisms are found, such as freeze, low pH and high temperature. These conditions are unfavorable to bacterial vegetative cells. However, spore-forming bacteria can survive to some of these conditions. Tolerance to low pH and high temperature are important tools for bacteria to survive in such conditions[2]. In this study, spores of *B. subtilis* isolated from a commercial fruit-juice added soy beverage showed high ability to survive in high temperatures and low pH.

The good manufacturing practices in the food industry are based on cleaning and organization measures to prevent accidents at work, optimize the chain production, avoid food contamination and assure safety and quality[13]. Errors in any step of the food production, in cleaning surfaces, materials or ingredients, may lead to food contamination. Also, preservative methods are employed to eliminate contaminants or to reduce at acceptable levels without harming the nutritional and organoleptic characteristics. Some of these methods are not effective against bacterial spores and the misapplication of a method may allow spore survival[2]. Furthermore, the utilization of non-sanitized or non-sterilized packages may be the

origin of microbial contamination[13]. The presence of *B. subtillis* in the fruit juice-added soy beverage may be a consequence of failure to execute the good manufacturing practices and in to applicate a preservative method during this beverage chain production.

Bacillus subtilis has optimal growth on neutral pH. However, this bacterium alters metabolic pathways to survive in low pH conditions. According to Enany[14], *B. subtilis* remolds its metabolism to overproduce ATP and support growth and survival under acidic conditions. Another consequence of this metabolism remodeling is the elevation of the NAD^+/NADPH ratio. Proton pumps, like H+-ATPase, are involved in this process as a trial of the bacterium to maintain the pH homeostasis. Biofilm formation, alteration of cell membrane and repair of macromolecules are also related to the acid tolerance of bacterium[15]. These mechanisms are important for *B. subtilis* to survive in low pH foods.

Thermal resistance has been described in several studies about bacteria that contaminate food[2,13,16]. This thermal resistance is related to survival mechanisms of adaptive stress that increase the expression of heat shock-proteins that protect spore-forming cells[17]. This thermal resistance has been widely studied once thermal treatments, such as pasteurization and Ultra-High Temperatures, should kill these cells, but in some cases it does not happen[2,13].

The *B. subtilis* isolated from fruit juice-added soy beverage exhibited tolerance to high temperature. Adekanmi et al.[18] submitted *Bacillus subtilis* W4m isolated from "Tsire-suya", a Nigerian meat product, to high temperatures, and obtained a D-value of 7.12 min at 60°C, and a Z-value of 58.82°C. Spores of *B. subtillis* strain PY79 submitted to 100°C and 110°C exhibited D-values of 8 min and approximately 0.7 min, respectively[19]. *Bacillus atrophaeus* spores were submitted to heat treatment at 105°C and exhibited a D-value of 25.6 min (±3.2min)[20]. Compared to our results, spores of *B. atrophaeus* were even more tolerant to high temperature than *B. subtilis* spores.

CONCLUSIONS

Our results suggest that the spores of *B. subtilis* isolated from fruit juice-added soy beverage are tolerant to low pH and high temperature. Analysis of exposure to low pH suggests that *B. subtilis* spores may survive in stressful conditions without geminating. It is possible that these spores were present in some point of the beverage chain production, contaminating the final product.

REFERENCES

1) Logan, A; Vos, P. 2015. *Bacillus*. Bergey's manual of systematics of archaea and bacteria. 1-163. DOI: https://doi.org/10.1002/9781118960608.gbm00530

2) Voundi, SO; Nyegue, M; Bougnom, BP; Etoa, FX. The Problem of Spore-Forming Bacteria in Food Preservation and Tentative Solutions. In: Singh OM. ed. Food borne Pathogens and Antibiotic Resistance. Nova Jersey: John Wiley & Sons. 2017, 139 p.

3) Karaman, AD; Alvarez, VB. Microbiology of evaporated, condensed and powdered milk. In: Ozer B; Evrendilek GA. ed. Dairy Microbiology and Biochemistry: Recent Developments. Boca Raton: CRC Press. 2014, 271 p.

4) Moschonas, G; Lianou, A; Nychas, GJE; Panagou, EZ. 2021. Spoilage potential of *Bacillus subtilis* in a neutral-pH dairy dessert. Food Microbiology. (95): 103715. DOI: https://doi.org/10.1016/j.fm.2020.103715

5) Logan, NA. 2012. *Bacillus* and relatives in foodborne illness. Journal of applied microbiology. 112 (3): 417-429. DOI: https://doi.org/10.1111/j.1365-2672.2011.05204.x

6) Hu, Q; Fang, Y; Zhu, J; Xu, W; Zhu, K. 2021. Characterization of *Bacillus* Species from Market Foods in Beijing, China. Processes. 9 (5): 866. DOI: https://doi.org/10.3390/pr9050866

7) Sabbaghi, A; Ghaemi, A. Molecular Adjuvants for DNA Vaccines: Application, Design, Preparation, and Formulation. In: Sousa Â. ed. DNA Vaccines. New York: Humana. 2021, 87-112 p. DOI: https://doi.org/10.1007/978-1-0716-0872-2_5

8) Suzuki, M; Rappé, MS; Giovannoni, SJ. 1998. Kinetic bias in estimates of coastal picoplankton community structure obtained by measurements of small-subunit rRNA gene PCR amplicon length heterogeneity. Applied and environmental microbiology. 64 (11): 4522-4529. DOI: 10.1128/AEM.64.11.4522-4529.1998

9) Altschul, SF; Gish, W; Miller, W; Myers, EW; Lipman, DJ. 1990. Basic local alignmentsearch tool. Journalof molecular biology. 215 (3): 403-410. DOI: https://doi.org/10.1016/S0022-2836(05)80360-2

10) Schinköthe, J; Scheinemann, HA; Diederich, S; Freese, H; Eschbaumer, M; Teifke, JP; Reiche, S. 2021. Airborne Disinfection by Dry Fogging Efficiently Inactivates Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), Mycobacteria, and Bacterial Spores and Shows Limitations of Commercial Spore Carriers. Applied and Environmental Microbiology. 87 (3). DOI: 10.1128/AEM.02019-20

11) Dash, KK; Deka, P. Thermal Processing of food. In: Dash, KK; Chakraborty, S. ed. Food Processing: Advances in Thermal Technologies. Boca Raton: CRC Press. 2021, 91 p.

12) Rossi, EM; Beilke, L; Barreto, JF. 2018. Microbial contamination and good manufacturing practices in school kitchen. Journal of food safety. 38 (1): e12417. DOI: https://doi.org/10.1111/jfs.12417

13) Sharif, ZIM. Mustapha, FA; Jai, J; Yusof, NM; Zaki, NAM. 2017. Review on methods for preservation and natural preservatives for extending the food longevity. Chemical Engineering Research Bulletin. 19: 145-153. DOI:https://doi.org/10.3329/cerb.v19i0.33809

14) Enany, S. 2020. Impact of Low pH on Microbial Growth Rate, ATP Production, and NADH to NAD ratio. Egyptian Journal of Medical Microbiology.29 (3): 121-128. DOI: DOI: https://doi.org/10.51429/EJMM29314

15) Guan, N; Liu, L. 2020. Microbial response to acid stress: mechanisms and applications. Applied microbiology and biotechnology.104 (1): 51-65. DOI: https://doi.org/10.1007/s00253-019-10226-1

16) Gharib, A; El-Hamid, A; Ayob, E. 2020.Physiological and Molecular Studies on Thermotolerance of *Bacillus cereus* Isolated from Some Dairy Products and Fast Foods. Zagazig Veterinary Journal. 48 (4): 354-365. DOI: 10.21608/zvjz.2020.35504.1113

17) Schäfer, H; Beckert, B; Frese, CK; Steinchen, W; Nuss, AM; Beckstette, M; Hantke, I; Driller, k; Sudzinová, P; Krásný, L; Kaever, V; Dersch, P; Bange, NW; Turgay, K. 2020. The alarmones (p) ppGpp are part of the heat shock response of *Bacillus subtilis*. PLoS genetics. 16 (3): e1008275. DOI: https://doi.org/10.1371/journal.pgen.1008275

18) Adekanmi, AA; Dapo, OA; Adekanmi, AS; Adekunle, MT. 2020. Heat Resistance of *Bacillus* Species Isolated From Tsire-Suya. International Journal of Academic and Applied Research. 4 (5): 31-46.

19) Krajčíková, D; Bugárová, V; Barák, I. 2021.Interactions of *Bacillus subtilis* Basement Spore Coat Layer Proteins. Microorganisms. 9 (2): 285. DOI: https://doi.org/10.3390/microorganisms9020285

20) Yung, PT; Lester, E; Ponce, A. 2020. Quantitative and fast Sterility Assurance testing of Surfaces by enumeration of Germinable endospores. Scientific reports. 10 (1): 1-10. DOI: https://doi.org/10.1038/s41598-019-57175-3