







Correlation Between Heat Resistance and Biofilm Formation of *Bacillus* Strains' Spores Isolated From Raw Cow's Milk



Bao Thy Vuong^{1,*} , Bich Hanh Bui Thi^{1,*} , Thanh Huy Tran Huu¹ , The Vinh Bui¹ , Thi-Kim-Dung Hoang²  and Khoa Anh Ton¹ 

¹Faculty of Health Sciences, Mekong University, Phu Quoi, Vinh Long, Vietnam

²Department of Organic and Environmental Technology, Institute of Advanced Technology - Vietnam Academy of Science and Technology, Ho Chi Minh City, Viet Nam

Abstract:

Introduction: *Bacillus* spp. pose a critical challenge to the dairy industry due to their ability to form highly heat-resistant endospores and robust biofilms on processing surfaces, leading to recontamination and food safety risks. This study aimed to investigate the correlation between the heat resistance of spores and the biofilm formation capacity of seven *Bacillus* strains isolated from raw cow's milk in the Mekong Delta, Vietnam.

Methods: Thermal resistance (D-values) was determined by treating spores at 80, 85, 90, and 95 °C for intervals of 12, 15, 20, 25, and 30 minutes. Biofilm formation was quantified using the crystal violet assay in 96-well plates via OD₅₇₀ measurements. Additionally, phylogenetic relationships were elucidated through 16S rRNA gene sequencing and compared with GenBank sequences to identify the isolates.

Results: A significant positive correlation was observed between biofilm density and spore heat resistance, with an R-squared (R²) value of = 0.881 ($p = 0.00174$). Strains with higher OD₅₇₀ values exhibited significantly greater D₉₅ values. Notably, strain TGS11.1 showed the highest thermal resistance (D₉₅ = 11.37 minutes) and the strongest biofilm-forming ability (OD₅₇₀ = 0.3196). Phylogenetic analysis indicated a high degree of genetic similarity between these isolates and *Bacillus cereus*.

Discussion: These findings reveal a “double risk” from indigenous *Bacillus* spp. strains, where the ability to persist via biofilms and survive thermal processing through resistant spores occurs simultaneously. This synergy complicates sterilization protocols and suggests that biofilm-forming capacity could potentially serve as an indicator for thermal persistence in dairy processing environments.

Conclusion: The study highlights a clear link between biofilm formation and spore heat resistance in *Bacillus* spp. These results underscore the necessity for more stringent interventions and offer practical guidance for optimizing sterilization practices to ensure the safety of dairy products.

Keywords: *Bacillus cereus*, Biofilm matrix, Heat resistance, D_r-value, Raw cow's milk, Linear regression, Mekong Delta.

© 2026 The Author(s). Published by Bentham Open.

This is an open access article distributed under the terms of the Creative Commons Attribution 4.0 International Public License (CC-BY 4.0), a copy of which is available at: <https://creativecommons.org/licenses/by/4.0/legalcode>. This license permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

*Address correspondence to these authors at the Faculty of Health Sciences, Mekong University, Phu Quoi, Vinh Long, Vietnam; E-mails: buihibichhanh@mku.edu.vn and vuongbaothy@mku.edu.vn

Cite as: Vuong B, Thi B, Huu T, Bui T, Hoang T, Ton K. Correlation Between Heat Resistance and Biofilm Formation of *Bacillus* Strains' Spores Isolated From Raw Cow's Milk. Open Microbiol J, 2026; 20: e18742858493934. <http://dx.doi.org/10.2174/0118742858493934260524112807>



Received: April 13, 2026
Revised: May 05, 2026
Accepted: May 08, 2026
Published: June 02, 2026



Send Orders for Reprints to
reprints@benthamscience.net

1. INTRODUCTION

The dairy industry plays a vital role in global agricultural economies and nutritional security. However, maintaining commercial sterility and sensory quality of milk products remains a major challenge due to contamination by *Bacillus* spp. strains, a group of bacteria capable of forming heat-resistant endospores and biofilms on the surfaces of processing equipment [1]. A recent systematic review reported a global prevalence of this Gram-positive genus in milk and dairy products of 11.8%, with the highest rate in Asia (15.4%) [2]. Their spores can survive thermal treatments due to multilayered structures; under favorable conditions, spores germinate, regenerate into vegetative cells, and proliferate, leading to product spoilage and food safety risks [3, 4]. The microbiological hazard is further exacerbated when these bacteria adhere to stainless steel pipelines or tanks and form complex biofilms, creating persistent reservoirs of recontamination that are difficult to eliminate with conventional cleaning procedures [5]. Although their importance has been recognized, most studies have evaluated heat resistance and biofilm formation as separate physiological traits [6]. Other investigations have highlighted the protective role of biofilm against environmental stresses, including high temperature, disinfectants, and oxidative stress [7-9]. The microbiological risk in dairy plants increases substantially when *Bacillus* spp. strains adhere and develop biofilms within stainless steel processing systems. Biofilm not only serves as a reservoir for recontamination but also functions as a protective shield, enabling spores to resist adverse factors such as cleaning chemicals and thermal stress [10]. Nevertheless, experimental studies that simultaneously assess and establish a direct correlation between thermal resistance (D_T value) and biofilm formation in the same bacterial strain remain limited. In particular, convincing evidence is still lacking to confirm whether the most heat-resistant strains are also the strongest biofilm formers under identical conditions [11]. Therefore, evaluating the correlation between heat resistance and biofilm formation in *Bacillus* spp. is not only academically relevant but also critical for food safety risk assessment and the development of effective control strategies in the dairy industry. In Vietnam, especially in the Mekong Delta region, data on this relationship in indigenous *Bacillus* strains isolated from raw milk remain scarce, representing a knowledge gap that needs to be addressed to optimize Cleaning-in-Place (CIP) procedures. This study was conducted to determine the correlation between heat resistance ability and biofilm spore-forming ability of seven *Bacillus* spp. strains isolated from raw cow's milk in the Mekong Delta. To achieve this objective, D_T values of spores were determined at four temperature levels (80, 85, 90, and 95°C). In parallel, biofilm formation was quantitatively assessed using the Crystal Violet staining method (OD_{570} measurement). The correlation between biofilm formation and spore heat resistance (represented by D_{95} values) was analyzed using linear regression modeling. In addition, genetic relationships and strain identification were clarified through 16S rRNA gene

sequencing. The findings of this study not only contribute to academic understanding of bacterial adaptation mechanisms but also provide practical evidence for establishing more effective Critical Control Points (CCPs), thereby enhancing food safety standards in the Vietnamese dairy industry.

2. MATERIALS AND METHODS

2.1. Sample Collection

Six raw cow's milk samples were collected in the morning between 7:00 and 9:00 a.m. from dairy farms located in several provinces of the Mekong Delta, Vietnam. Each sample was randomly obtained in sterile plastic containers, kept under refrigeration, and transported to the laboratory within a maximum of 4 hours. Upon arrival, samples were stored at 4 °C until bacterial isolation was performed.

2.2. Isolation and Identification of Bacterial Strains

A 150 mL aliquot of raw milk was heat-treated at 80 °C for 12 min in a thermostatic water bath to eliminate vegetative cells, followed by immediate cooling in an ice-water bath [12]. Subsequently, the bacterial isolation procedure was assessed following the method of a study [13], with modifications: 1 mL of diluted sample was spread-plated onto Luria Bertani (LB) agar medium (10 g/L Peptone, 5 g/L Yeast extract, 10 g/L NaCl, 20 g/L Agar) and incubated at 37 °C for 24 h. Colonies were observed, and pure cultures were obtained by repeated streaking. Isolated strains were preserved in LB-glycerol (1:1) at -80 °C for subsequent experiments.

2.3. 16S rRNA-based Bacterial Identification

Pure bacterial isolates were subjected to Gram staining, motility testing, and catalase reaction following the guidelines of Bergey's Manual of Systematic Bacteriology [14]. Gram-positive, motile, and catalase-positive strains were selected for 16S rRNA gene sequencing to identify target species. The primer pair used for amplification was 27F (5'-AGAGTTTGATCCTGGC TCAG-3') and 1492R (3'-CGGTTACCTGTACGACTT-5'). The resulting 16S rRNA sequences were compared with available sequences in the NCBI GenBank database using the BLAST online tool (<https://blast.ncbi.nlm.nih.gov>). Based on BLAST results, phylogenetic trees were constructed, and scientific nomenclature of the isolates was determined.

2.4. Assessment of Thermal Tolerance of Bacterial Spores

Spores of target strains were produced by incubating cultures in 1% (w/v) peptone solution at 37 °C for 14 days, followed by heat treatment at 80 °C for 10 min to eliminate vegetative cells. Spore suspensions were stored at 4 °C until use. Thermal inactivation was performed according to the method described by Lin *et al.* (2022) [15], with modifications: 2.5 mL of spore suspension was exposed to 80, 85, 90, or 95 °C in a water bath for 12-30 min, then rapidly cooled in ice. Surviving spores were quantified by

plating 1.0 mL of heated suspension on LB agar, incubating at 37 °C for 48 h, and enumerating CFU using the plate count method on PCA. Mean values were calculated from three independent replicates.

2.5. D-value Calculation

The heat resistance of each strain was assessed by determining the D_T value (min), calculated from the number of surviving spores after thermal treatment using the following formula: $D_T = \frac{t}{\log N - \log N_0}$. Where: + D_T : Decimal reduction time at temperature T. + N: The number of microorganisms in the product at time t (CFU/mL) + N_0 : The initial number of microorganisms (CFU/mL) + t: Heating time (minutes).

2.6. Quantitative Biofilm Formation

The biofilm-forming ability of bacterial strains was assessed following the method described by Stepanović *et al.* (2007) [16], with modifications. Cultures were grown in the TSB + Y medium (30 g/L Tryptone Soy Broth; 5 g/L Yeast extract) at 200 rpm for 12 h, diluted 1:10 in fresh TSB, and incubated statically at 37 °C for 12 h. Aliquots (200 μ L) were inoculated into sterile 96-well microtiter plates and incubated statically at 37 °C for 48 h; wells containing only medium served as negative controls. After incubation, non-adherent cells were removed, wells were washed with PBS, biofilms were fixed with methanol, stained with 1% crystal violet, and the bound dye was solubilized with 95% ethanol. Optical density (OD_{570}) was measured to quantify biofilm biomass. Biofilm-forming ability was classified as follows: non-biofilm producer ($OD_{\text{Sample}} \leq OD_C$), weak biofilm producer ($OD_C < OD_{\text{Sample}} \leq 2OD_C$), moderate biofilm producer ($2OD_C < OD_{\text{Sample}} \leq 4OD_C$), and strong biofilm producer ($OD_{\text{Sample}} > 4OD_C$), where $OD_C = OD_{\text{Control}} + 3 \times SD$ (OD_{Control} : mean absorbance of negative control wells; SD: standard deviation).

2.7. Morphological Analysis of *Bacillus* Biofilm by Scanning Electron Microscopy

Biofilm samples of *Bacillus* were diluted by mixing 1 drop of suspension in 1 mL deionized water, and a drop of the diluted sample was placed on a clean silicon wafer and air-dried at room temperature to minimize charging during SEM analysis. Morphological characterization was performed using a field-emission scanning electron microscope (FE-SEM, Hitachi S-4800) operated at 10.0 kV with a working distance of ~ 10.2 mm in secondary electron mode. Representative images were acquired at magnifications of 2000 \times and 5000 \times to visualize both the overall biofilm architecture and the rod-shaped morphology of *Bacillus* cells.

2.8. Biofilm Cultivation and Biomass Harvesting

Four high-biofilm-forming strains were cultured in Brain Heart Infusion (BHI; 10.0 g/L proteose peptone, 2.0 g/L dextrose, 5.0 g/L NaCl, 2.5 g/L Na_2HPO_4). For large-scale production, 800 μ L of bacterial suspension was inoculated into 150-mm Petri dishes containing 80 mL of BHI (1:100 ratio), with 90 replicates per strain, and

incubated statically at 37 °C for 72 h to allow mature pellicle biofilm formation. After incubation, planktonic cells were removed, biofilms were rinsed three times with sterile PBS, harvested using a cell scraper, resuspended in 2 mL PBS, and pooled into 50-mL centrifuge tubes. Biomass was concentrated by centrifugation at 10,000 rpm for 10 min at 4 °C, and the final yield was quantified by measuring wet weight (Mwet) for standardized analyses.

2.9. Statistical Analysis

Data were processed using Microsoft Excel 2016 and GraphPad Prism software. A one-way ANOVA followed by Tukey's post hoc test was applied to compare mean values, with significance set at $p < 0.05$. The correlation between OD_{570} and D_{95} values was evaluated using Spearman's rank correlation and a simple linear regression model ($y = ax + b$).

3. RESULTS AND DISCUSSION

3.1. Isolation and Morphological Characterization of *Bacillus* spp

A total of seven spore-forming bacterial strains were successfully isolated from six raw cow's milk samples collected across dairy stations in the Mekong Delta, Vietnam. The isolates exhibited typical phenotypic characteristics of the genus *Bacillus*: colonies were predominantly opaque white to ivory, with diameters ranging from 1 to 3 mm. Morphological variations included circular to irregular shapes, rough surfaces, and smooth or serrated edges (Fig. 1). Gram staining and biochemical screening further revealed that all isolates were Gram-positive, short rod-shaped cells, and demonstrated motility and catalase-positive activity. To confirm their taxonomic status, 16S rRNA gene sequence analysis was performed, which definitively identified all seven strains as belonging to the genus *Bacillus*. These isolates were subsequently utilized for thermal resistance and biofilm formation assays.



Fig. (1). The colony morphology of the isolated strain after 24 hours incubation on LB agar.

3.2. Heat Resistance of *Bacillus* spp. Spores

To evaluate the heat resistance of spores from seven *Bacillus* spp. strains isolated from raw milk, decimal reduction times (D_T values, in minutes) were determined in peptone solution across a temperature range of 80–95 °C. As shown in Fig. (2), a clear inverse relationship between

treatment temperature and spore survival was observed: D_T values consistently decreased with increasing temperature across all strains. For any given *Bacillus* strain, higher treatment temperatures resulted in lower D_T values, indicating reduced thermal resistance. One-way ANOVA confirmed statistically significant differences among strains ($p < 0.001$). Notably, *Bacillus* TGS11.1 and *Bacillus* CTS13.1 exhibited statistically greater thermal stability than the other five strains at all tested temperatures. The thermal resistance of seven *Bacillus* spp. strains in peptone medium at 80–95 °C was evaluated based on D_T values (min), with data representing mean values from three independent replicates. One-way ANOVA followed by Tukey's test indicated significant differences among strains ($p < 0.001$). Notably, strains TGS11.1 and CTS13.1 consistently exhibited higher thermal stability than the other strains across all tested temperatures. At the highest treatment temperature (95 °C), spore heat resistance is shown in Fig. (3). Mean D_{95} values (\pm SD) ranged from 11.37 ± 0.45 min for *Bacillus* strain TGS11.1 to 0 min for strain CTS11.3, indicating clear stratification of thermal resistance among strains ($p < 0.001$). Error bars represent standard deviations from three independent replicates, confirming the reliability of the data. D_{95} values represent the time required for a 90% reduction of spore populations at 95 °C in peptone medium. Figure 3 shows mean values from triplicate experiments, with error bars indicating standard deviations. A gradient of heat resistance was observed ($p < 0.001$), ranging from the most resistant strain TGS11.1 (11.37 ± 0.45 min) to the most sensitive strain CTS11.3 (0 min). These values fall within previously reported ranges: comparable to 7.10–10.40 min for *Bacillus cereus* from milk in Cameroon [17], but lower than the 24 min or broader 1.50–36.00 min range documented for foodborne strains [18]. Thus, the strains examined here exhibit moderate heat resistance, lower than certain pathogenic isolates yet consistent with literature values. The observed resistance is attributed to spore-specific structural and biochemical features, including a thick cortex, high Ca-DPA content, DNA-protective SASPs, and a dehydrated core, which collectively reduce protein denaturation and DNA damage under heat stress [19]. The thermal resistance of spores from the seven *Bacillus* isolates was quantified using decimal reduction times (D_T values) in peptone solution across a temperature range of 80–95 °C. As illustrated in Fig. (2), a consistent inverse relationship was observed between treatment temperature and spore survival, with D_T values significantly decreasing as temperatures increased for all strains. Statistical analysis using one-way ANOVA followed by Tukey's post-hoc test confirmed significant differences in thermal stability among the isolates ($p < 0.001$). Notably, strains TGS11.1 and CTS13.1 consistently exhibited the highest thermal

stability across the entire temperature range tested. At the maximum treatment temperature of 95 °C (Fig. 3), the isolates displayed a clear gradient of heat resistance. The mean D_{95} values ranged from 11.37 ± 0.45 min for the most resistant strain (TGS11.1) to 0 min for the most sensitive (CTS11.3). These D_{95} values are comparable to previously reported ranges for *Bacillus cereus* isolated from milk (e.g., 7.10–10.40 min in Cameroon [17]). However, they remain lower than the higher thresholds documented for certain foodborne pathogenic strains [18]. Overall, the isolates examined in this study demonstrate a moderate level of heat resistance, which is consistent with the existing literature on dairy-related *Bacillus* environmental isolates.

3.3. Assessment of Biofilm Formation by Seven *Bacillus* spp. Strains using the Crystal Violet Assay

The biofilm-forming ability of seven *Bacillus* strains isolated from fresh cow's milk was evaluated using the CV staining method on sterile 96-well plates, followed by measuring optical density at 570 nm. Qualitative and quantitative results (Figs. 4 and 5) indicated that all tested strains formed biofilms at varying levels. Biofilm biomass was quantified using the crystal violet assay, with results expressed as mean OD_{570} values from three independent experiments performed in triplicate (error bars = SD). One-way ANOVA revealed significant differences among strains ($p < 0.001$). Based on OD thresholds, strains were classified as strong biofilm formers (TGS11.1, $OD_{570} > 4OD_c$), moderate formers (CTS13.1, TGS33.2, TGS21.2, CTS11.3, LAS23.1, OD_{570} between $2OD_c$ and $4OD_c$), and weak formers (LAS11.1, $OD_{570} \leq 2OD_c$). Tukey's post-hoc test ($p < 0.05$) confirmed significant differences between the strong (TGS11.1) and weak (LAS11.1) groups, whereas the moderate group did not differ significantly from either. The threshold OD_c value was determined to be 0.0704. Based on this threshold, biofilm formation was classified as weak, moderate, or strong. Strain LAS23.1 was identified as a weak biofilm producer ($OD_{570} < 2OD_c$). Five strains CTS11.3, CTS13.1, LAS11.1, TGS21.2, and TGS33.2 were classified as moderate producers (OD_{570} around $2OD_c$). Strain TGS11.1 exhibited the strongest biofilm formation, with a mean OD value of $0.3196 \pm$ SD, approximately 4.5-fold higher than OD_c . One-way ANOVA revealed no statistically significant differences in OD_{570} values among strains ($p > 0.05$). Visual observations supported the quantitative results. Crystal violet staining of 96-well microtiter plates (Fig. 5A) and the inner surfaces of microcentrifuge tubes (Fig. 5B) showed distinct differences in staining intensity. Strain TGS11.1 retained the darkest violet color, consistent with its highest OD value, whereas LAS23.1 exhibited only faint staining, reflecting low biofilm biomass. All experiments were performed in triplicate to ensure reproducibility.

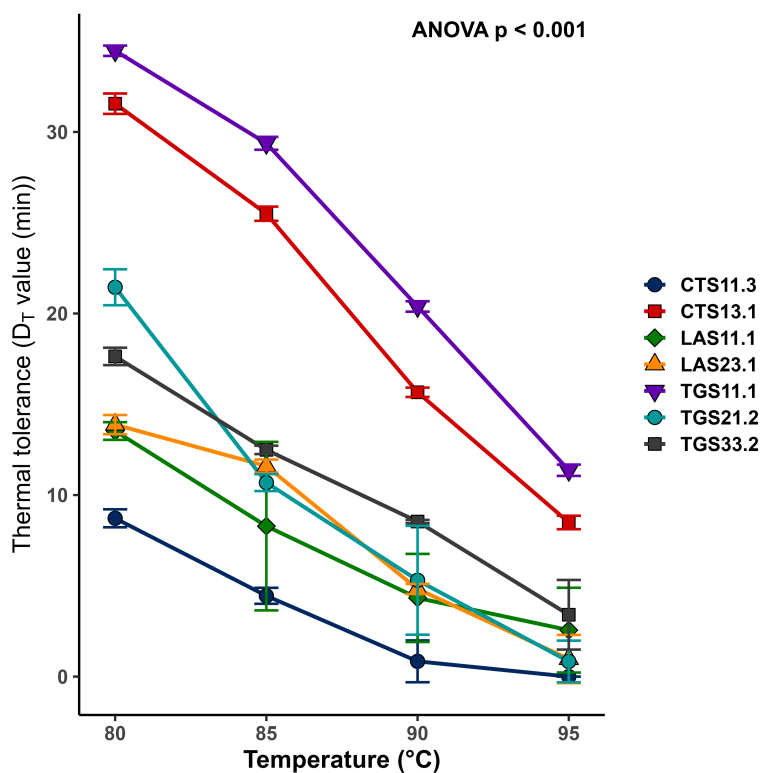


Fig. (2). Heat resistance profiles of seven Bacillus strains' spores isolated from raw milk. The heat resistance of seven Bacillus strains in peptone medium across the temperature range of 80–95 °C was determined by DT values (minutes). Data points represent mean values from three independent replicates. One-way ANOVA analysis revealed statistically significant differences in thermal resistance among the strains ($P < 0.001$). Notably, Bacillus strain TGS11.1 and Bacillus strain CTS13.1 consistently exhibited higher thermal stability compared to the other strains at the same tested temperatures.

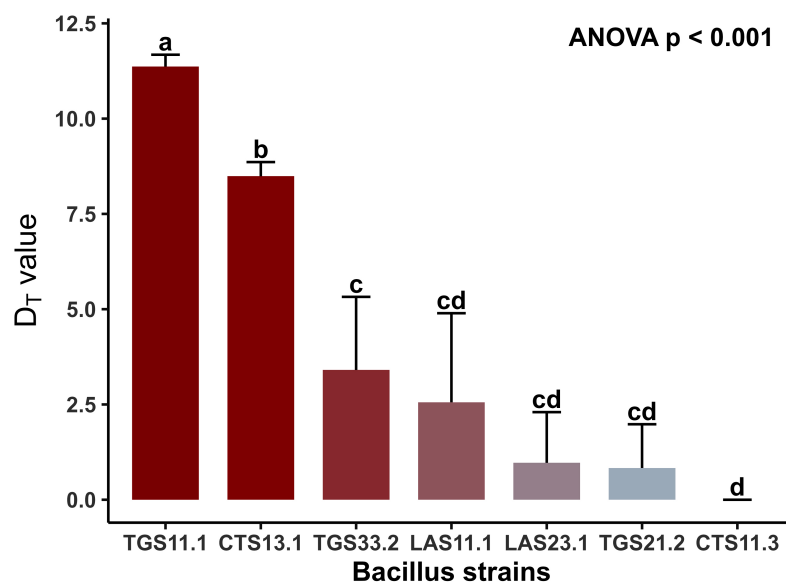


Fig. (3). Heat resistance (DT value) of spores of seven Bacillus spp. strains at 95 °C. The D₉₅ values (minutes) represent the time required to reduce the spore population by 90% at a constant temperature of 95°C in peptone medium. Bars represent the mean values of triplicate experiments, with error bars indicating the standard deviation (SD). Different shades of red are used to highlight the gradient of heat resistance, from the most resistant (TGS.11.1) to the most sensitive (CTS.11.3) strains ($p < 0.001$) at 95 °C.

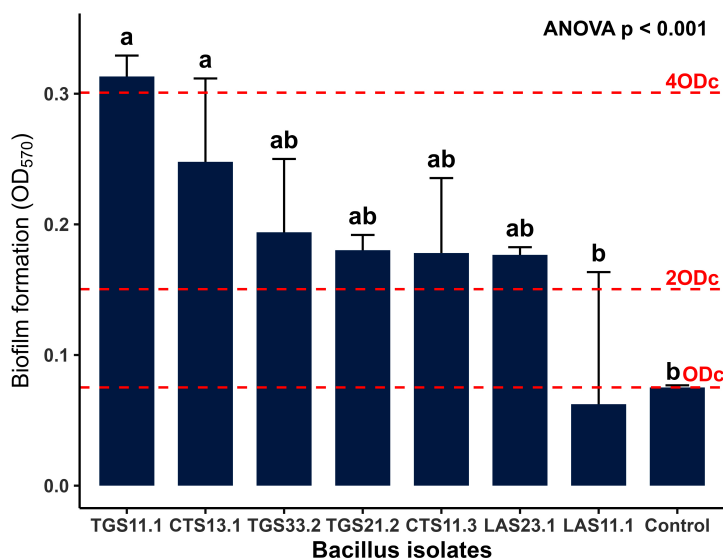


Fig. (4). Biofilm formation profiles of seven *Bacillus* strains isolated from raw cow milk. Biofilm biomass was quantified using the crystal violet assay, with results expressed as the mean optical density at 570 nm (OD_{570}). Data points represent the mean values from three independent experiments performed in triplicate, with error bars indicating the standard deviation (SD). Statistical analysis using one-way ANOVA revealed significant differences in biofilm-forming ability among the strains ($P < 0.001$). Different lowercase letters above the bars denote statistically significant differences between specific strains as determined by Tukey's post-hoc test ($p < 0.05$).

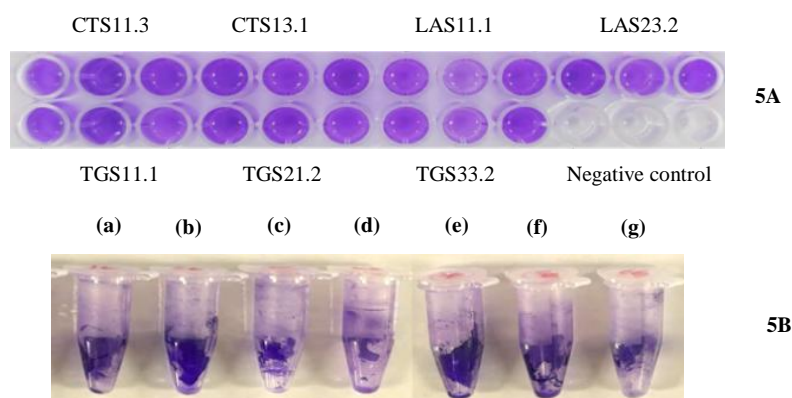


Fig. (5). Qualitative assessment of biofilm formation by seven *Bacillus* isolates. **(5A)** Representative 96-well microtiter plate following crystal violet (CV) staining, illustrating biofilm biomass distribution across seven *Bacillus* spp. strains. **(5B)** CV-stained biofilms formed on the inner surfaces of microcentrifuge tubes, showing strain-specific differences in dye retention. Tubes correspond to strains (a) CTS11.3, (b) CTS13.1, (c) LAS11.1, (d) LAS23.1, (e) TGS11.1, (f) TGS21.2, and (g) TGS33.2, with the intensity of blue/purple coloration reflecting relative biofilm matrix abundance. Experiments were performed in triplicate to ensure reproducibility.

3.4. Biofilm Morphology of the *Bacillus* Strain TGS11.1 observed by Scanning Electron Microscopy (SEM)

Direct visualization of biofilm architecture in the most heat-resistant strain, the *Bacillus* strain TGS11.1, was conducted by using SEM. The results revealed that this strain was capable of forming a highly robust and complex biofilm matrix. At 5,000 \times magnification, bacterial cells displayed the characteristic rod-shaped morphology of the

genus *Bacillus*, with uniform size and densely packed arrangements forming multiple overlapping layers (Fig. 6A). At 2,000 \times magnification, bacterial cells were observed to be densely packed and overlapping, forming a three-dimensional structure. This arrangement demonstrated strong intercellular adhesion, which supports the maintenance of a stable biofilm architecture. Perhaps the kind of structural matrix helps protect the bacterial community against external influences (Fig. 6B-D).

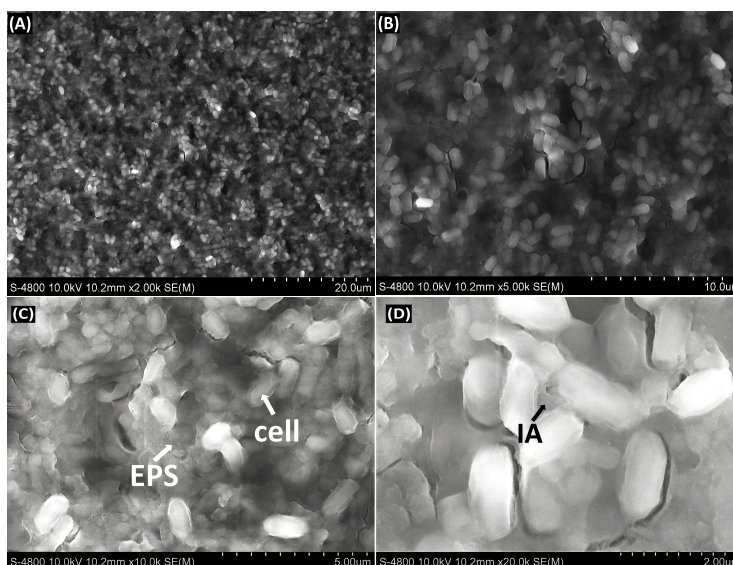


Fig. (6). Scanning electron microscopy (SEM) micrographs illustrating the robust 3D biofilm architecture of *Bacillus* sp. strain TGS11.1. (A-B) Overview at 2,000 \times and 5,000 \times magnification showing a dense, multi-layered 3D structure. (C-D) Detailed microstructure at 10,000 \times and 20,000 \times magnification. Arrows (EPS) film-like matrix encapsulating the bacterial community, forming a structural “thermal shield”, Arrows (Cell): Rod-shaped cells of strain TGS11.1 embedded within the matrix, Arrows (IA): Intercellular adhesion zones establishing a stable, resistant biofilm framework.

3.5. Correlation between Optical Density and Crude Biofilm Biomass

To verify whether OD_{570} measurements accurately reflected the physical accumulation of biofilm biomass, crude biofilm mass was quantified for two representative strains after 96 h of incubation. The strain TGS11.1, selected from the strong biofilm-forming group based on its highest OD_{570} value, and the strain CTS13.1, chosen from the moderate group with the highest OD_{570} value, were analyzed. The results, presented in Table 1 and Fig. (6), demonstrated that OD_{570} values corresponded well with the measured crude biofilm biomass, supporting the reliability of OD_{570} as a proxy for biofilm quantification. The physical mass of harvested biofilm was consistent with the optical density (OD_{570}) characteristics previously recorded. Specifically, the strain TGS11.1 produced the highest biomass at 3.54 g, approximately twice that of the strain CTS13.1 (1.76 g) (Table 1). Visual observations further supported these quantitative data: the thickness of the biofilm layer adhering to the Petri dish surface (Fig. 7A) and the volume of precipitate obtained after centrifugation (Fig. 7B) were proportional to the measured biomass. Biofilm formation in *Bacillus* spp. is influenced by multiple environmental conditions and intrinsic cellular properties. Among these, motility mediated by flagella plays a critical role in promoting the initial adhesion phase and facilitating subsequent biofilm development. Flagellar-driven movement enhances bacterial access to surfaces, increases opportunities for cell-surface interactions, and supports the establishment of a stable biofilm matrix. In addition, strains carrying dominant genotypes for exopolysaccharide (EPS)

synthesis, regulated by central factors such as Spo0A and the SinR/SinI system, typically produce thick, stable biofilms that develop in the form of submerged biofilms. These regulatory pathways promote EPS accumulation, reinforce structural integrity, and enable the persistence of biofilm communities under diverse environmental conditions. This phenotype-genotype divergence helps explain the persistence of *Bacillus cereus* in both environmental niches and food-processing lines. Strains with genetic profiles favoring exopolysaccharide synthesis and biofilm development can establish resilient communities, while their phenotypic traits, such as strong adhesion and multilayered biofilm structures, enable survival under stress conditions. Together, these features contribute to the long-term presence of *Bacillus cereus* in industrial settings, complicating eradication efforts and increasing the risk of contamination [20]. In this study, the strain TGS11.1 was chosen as the target strain for correlation analysis between biofilm formation capacity and heat resistance. This strain exhibited both the highest thermal tolerance and the strongest biofilm production among the isolates tested, making it an appropriate representative for evaluating the relationship between these two phenotypic traits.

Table 1. Crude biofilm biomass of two selected *Bacillus* spp. strains after 96 h of incubation.

Isolates	Incubation Time (hours)	Biofilm Biomass
TGS11.1	96	3.54 g
CTS13.1	96	1.76 g

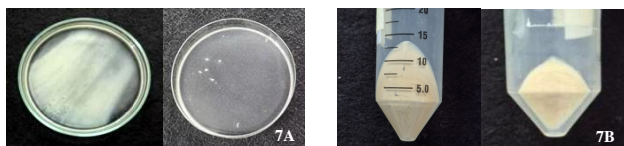


Fig. (7). Macro-structural characterization of biofilm formation by the most heat-resistant *Bacillus* sp. strains, TGS 11.1 and CTS 13.1. **(A)** Physical attachment of mature biofilms on the bottom surface of Petri dishes after 72 hours of incubation in Tryptic Soy Broth (TSB); **(B)** Macroscopic appearance of harvested crude biofilm biomass in 50 mL conical tubes following PBS washing and centrifugation.

3.6. Correlation between Heat Resistance and Biofilm Formation and its Practical Implications

To test the hypothesis that biofilm formation is associated with heat resistance in *Bacillus* spp., correlation and linear regression analyses were performed. A strong, positive, and statistically significant correlation was observed between OD_{570} and D_{95} , with Spearman's $\rho = 0.881$ ($p = 0.00174$). The scatter plot (Fig. 8) demonstrates that strains with greater biofilm biomass exhibited spores with stronger thermal resistance. Linear regression ($y = 0.0127x + 0.1632$) yielded $R^2 = 0.881$, indicating that biofilm formation

explained ~88% of the variation in heat resistance. The slope ($a = 0.0127$) suggests that each unit increase in OD_{570} extended spore survival at 95°C by 0.0127 min. Despite strain-specific variation (e.g., CTS11.3, TGS21.1), the relationship remained statistically significant ($p < 0.01$). These findings provide experimental evidence that the EPS matrix acts as a protective barrier against heat stress and support the predictive value of biofilm development for spore thermal resistance.

3.7. Genetic Relationships of the Seven *Bacillus* spp. Strains with Bacterial Strains

A 16S rRNA-based phylogenetic tree was constructed to determine the taxonomic position of *Bacillus* isolates from raw cow milk, with *Escherichia coli* NBRC 102203 as the outgroup (Fig. 9). The seven isolates (highlighted in red) formed a monophyletic clade with maximum bootstrap support (100%), confirming their affiliation with the genus *Bacillus*. Within the genus, the isolates tended to cluster with the *Bacillus cereus* group (e.g., *Bacillus cereus*, *Bacillus paramycoides*, *Bacillus albus*, *Bacillus pacificus*), though bootstrap values $< 50\%$ prevented definitive species-level identification. Overall, the results indicate close evolutionary relatedness among the isolates, while also highlighting the limited resolution of 16S rRNA sequencing for distinguishing species boundaries within the *Bacillus cereus* group.

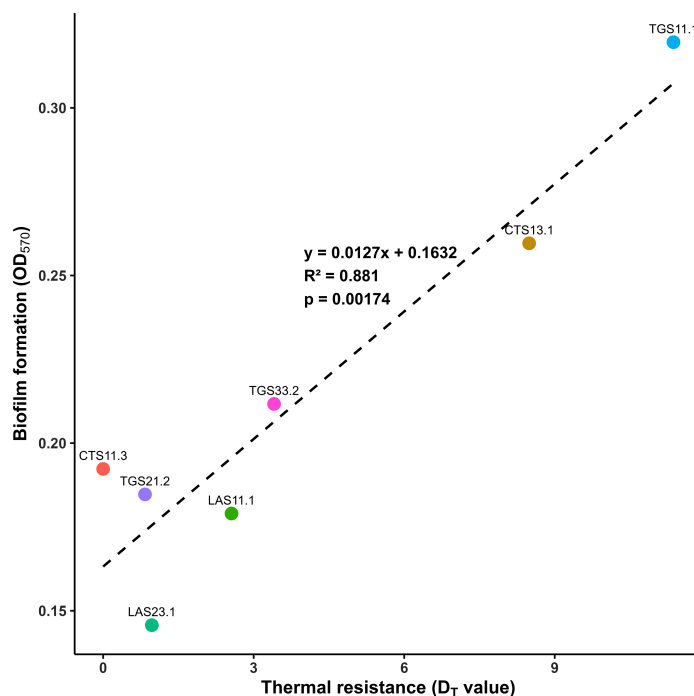


Fig. (8). Correlation between biofilm formation and thermal resistance of the *Bacillus* spp. spores. A scatter plot illustrates the linear relationship between biofilm-forming capacity (OD_{570}) and heat resistance (D_{95}) across seven isolates. The regression line ($y = 0.0127x + 0.1632$) yielded $R^2 = 0.881$ and Pearson's $r = 0.938$, indicating a strong, statistically significant positive correlation ($p = 0.00174$). Data points represent mean values from three independent replicates, with error bars denoting standard deviations.

collection, statistical analysis, and participated in manuscript revision; T.V.B.: Contributed to data analysis and assisted in manuscript revision.; T.K.D.H.: Provided support with equipment and infrastructure for the experiments; K.A.T: Supervised the project and provided critical review.

LIST OF ABBREVIATIONS

BHI	=	Brain Heart Infusion
CCPs	=	Critical Control Points
CFU	=	Colony Forming Unit
CIP	=	Clean in Place
CV	=	Crystal Violet
DPA	=	Dipicolinic acid
DT	=	Decimal reduction Time
EPS	=	Exopolysaccharide
LB	=	Luria Bertani
OD	=	Optical Density
PBS	=	Phosphate Buffered Saline
PCA	=	Plate Count Agar
SASPs	=	Small Acid-Soluble Spore Proteins
SEM	=	Scanning Electron Microscopy
Spp.	=	species pluralis
TSB	=	Tryptic Soy Broth
UHT	=	Ultra-High Temperature processing
Y	=	Yeast

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of the article is available from the corresponding authors [B.V. and B.H.B.T] upon reasonable request.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The authors would like to express their sincere gratitude to the Institute of Advanced Technology,

Vietnam, and the Academy of Science and Technology, Vietnam, for providing the laboratory facilities and technical support necessary to conduct this research.

REFERENCES

- [1] Medjahdi K, Didouh N, Araujo R. Pasteurized milk: A highlight on potential sources of contamination by aerobic Spore-Forming bacteria. *Food Control* 2025; 171: 111134. <http://dx.doi.org/10.1016/j.foodcont.2025.111134>
- [2] Sun T, Wang R, Sun Y, Zhang X, Ge C, Li Y. The global prevalence of *Bacillus* spp. in milk and dairy products: A systematic review and Meta-Analysis. *Foods* 2025; 14(15): 2599. <http://dx.doi.org/10.3390/foods14152599> PMID: 40807537
- [3] Tirloni E, Stella S, Celandroni F, Mazzantini D, Bernardi C, Ghelardi E. *Bacillus cereus* in dairy products and production plants. *Foods* 2022; 11(17): 2572. <http://dx.doi.org/10.3390/foods11172572> PMID: 36076758
- [4] Diarra C, Goetz C, Gagnon M, Roy D, Jean J. Biofilm formation by Heat-Resistant dairy bacteria: Multispecies biofilm model under static and dynamic conditions. *Appl Environ Microbiol* 2023; 89(10): e00713-23. <http://dx.doi.org/10.1128/aem.00713-23> PMID: 37732743
- [5] LaPointe G, Wilson T, Tarrah A, Gagnon M, Roy D. Biofilm formation in dairy: A food safety Concern—Microbial community tracking from dairy farm to factory; Insights on biofilm management for enhanced food safety and quality. *J Dairy Sci* 2025; 108(8): 8101-19. <http://dx.doi.org/10.3168/jds.2024-25397> PMID: 39788184
- [6] Pawluk AM, Kim D, Jin YH, Jeong KC, Mah JH. Biofilm-Associated heat resistance of *Bacillus cereus* spores *in vitro* and in a food model, Cheonggukjang jjigae. *Int J Food Microbiol* 2022; 363: 109505. <http://dx.doi.org/10.1016/j.ijfoodmicro.2021.109505> PMID: 34973549
- [7] Goetz C, Sanschagrin L, Jubinville E, Jacques M, Jean J. Biofilm formation in dairy: A food safety Concern—Recent progress in antibiofilm strategies in the dairy industry. *J Dairy Sci* 2025; 108(8): 8157-75. <http://dx.doi.org/10.3168/jds.2024-25554> PMID: 39603496
- [8] Liu Y, Zhang B, Wang S, Yang Z, Yuan L. Different risk potential of *bacillus cereus* to the dairy industry – keeping up with the old story. *Food Science of Animal Products* 2024; 2(4): 9240085. <http://dx.doi.org/10.26599/FSAP.2024.9240085>
- [9] Catania AM, Di Ciccio P, Ferrocino I, Civera T, Cannizzo FT, Dalmasso A. Evaluation of the Biofilm-Forming ability and molecular characterization of dairy *Bacillus* spp. isolates. *Front Cell Infect Microbiol* 2023; 13: 1229460. <http://dx.doi.org/10.3389/fcimb.2023.1229460> PMID: 37600945
- [10] Liang L, Wang P, Zhao X, *et al.* Metabolomics aided by machine learning decodes adaptive remodeling of *Bacillus* biofilms in response to pasteurization stress. *NPJ Sci Food* 2026; 10(1): 62. <http://dx.doi.org/10.1038/s41538-026-00712-y> PMID: 41559097
- [11] Kim S, Mah JH. Variation in heat resistance and biofilm formation of *Bacillus cereus* spores in various fermented soybean foods. *Int J Food Microbiol* 2025; 427: 110939. <http://dx.doi.org/10.1016/j.ijfoodmicro.2024.110939> PMID: 39437681
- [12] Ferrero A, Ferrero F, Casale M, *et al.* The impact of different temperatures on spore count and browning of UHT milk over a long storage period. *Int Dairy J* 2024; 155: 105948. <http://dx.doi.org/10.1016/j.idairyj.2024.105948>
- [13] Golnari M, Bahrami N, Milanian Z, *et al.* Isolation and characterization of novel *Bacillus* strains with superior probiotic potential: Comparative analysis and safety evaluation. *Sci Rep* 2024; 14(1): 1457. <http://dx.doi.org/10.1038/s41598-024-51823-z> PMID: 38228716
- [14] Amini-Rourani H, Hosseini-Abari A, Taherzadeh MJ. Degradation of pectin with probiotic candidate *Bacillus subtilis* HA1 to

- oligosaccharides with enhanced anticancer and antioxidant properties. *Int J Biol Macromol* 2025; 331(2): 148501. <http://dx.doi.org/10.1016/j.ijbiomac.2025.148501> PMID: 41135889
- [15] Lin T, Bian H, Sun Z, Wang X, Liu F, Wang D. Inactivation of *Clostridium perfringens* C1 spores by the combination of mild heat and lactic acid. *Foods* 2022; 11(23): 3771. <http://dx.doi.org/10.3390/foods11233771> PMID: 36496579
- [16] Stepanović S, Vuković D, Hola V, *et al.* Quantification of biofilm in microtiter plates: Overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *Acta Pathol Microbiol Scand Suppl* 2007; 115(8): 891-9. http://dx.doi.org/10.1111/j.1600-0463.2007.apm_630.x PMID: 17696944
- [17] Tatsinkou Fossi B, Tatah Kihla Akoachere J-F, Nchanji GT, Wanji S. Occurrence, heat and antibiotic resistance profile of *Bacillus cereus* isolated from raw cow and processed milk in Mezam Division, Cameroon. *Int J Dairy Technol* 2016; 70: 43-51. <http://dx.doi.org/10.1111/1471-0307.12315>
- [18] Griffiths MW, Schraft H. *Bacillus cereus* Food Poisoning. In: Dodd CER, Aldsworth TG, Stein RA, Cliver DO, Riemann HP, Eds. *Foodborne Diseases* (Third Edition). London, UK: Academic Press 2017; pp. 395-405. <http://dx.doi.org/10.1016/B978-0-12-385007-2.00020-6>
- [19] Setlow P, Christie G. What's new and notable in bacterial spore killing. *World J Microbiol Biotechnol* 2021; 37(8): 144. <http://dx.doi.org/10.1007/s11274-021-03108-0> PMID: 34351499
- [20] Ghosh B, Dhar J, Mukhopadhyay M, Bhattacharya D. *Bacillus cereus* biofilm: Implications for food and diseases. *Microbe* 2024; 4: 100129. <http://dx.doi.org/10.1016/j.microb.2024.100129>