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HEAT RESISTANCE TESTING OF *BACILLUS* SPP. SPORES ISOLATED FROM HOUSEHOLDS' RAW MILK IN PEPTONE MEDIA AND DAIRY PRODUCTS

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ABSTRACT

In dairy production, raw cow's milk is an ideal nutrient-rich environment for various microorganisms. The microbiota of raw milk is highly diversified, comprising not only spoilage and pathogenic microorganisms but also bacteria with potential technological relevance. The microbial content in milk is a critical factor in determining its quality, sensory characteristics, and the overall quality of dairy products. Additionally, a suitable heat treatment regime has been chosen to make safe dairy products while using less heat energy to process, resulting in reduced carbon emissions and minimal environmental impact. In this study, raw milk samples were randomly collected from household milk collection stations in the Mekong Delta, Vietnam. The samples were heat-treated at 80°C for 12 minutes and then incubated in peptone medium at 37°C for 24 hours. As a result, 30 bacterial strains capable of forming heat-resistant spores were isolated. Among them, 07 strains that produced high levels of extracellular lipase and protease were identified as *Bacillus* spp. based on morphological characteristics and biochemical properties according to Bergey's classification system. The heat resistance abilities of 07 strains' spores were tested in peptone medium at 100°C for different heat treatment durations; the results showed that only the TG11.1 strain survived after 30 minutes at 100°C of the heat treatment. Additionally, the TG11.1 strain exhibited the highest heat resistance in unsweetened milk, with a thermal death time (D-value) of 30 minutes at 100°C. This study offers valuable insights into the heat resistance of *Bacillus* spp. spores isolated from locally sourced raw cow's milk. It serves as a practical reference for small-scale dairy producers, enabling them to choose and apply suitable heat treatment methods tailored to different dairy products. By doing so, producers can ensure the safety and nutritional quality of their products while optimizing production costs. Furthermore, this approach contributes to the development of a more sustainable food system, aligning with global efforts to address environmental concerns and promote food security.

Key words: *Bacillus*, raw milk, spores, heat resistance, peptone media, nutritious milk

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INTRODUCTION

In Vietnam, smallholder dairy farms (SDFs) represent the most common farming system. According to a 2017 survey, there were approximately 28,695 SDFs, with an average of 20 or fewer cows per farm, accounting for 97% of the national dairy herd and 80% of fresh milk production. Vietnam's milk production has shown consistent growth, ranking sixth in Asia and second in the ASEAN region in 2018 [1]. The dairy industry plays a significant role in the economy and has received substantial government support, including interest rate subsidies for breed development, farm expansion, and technological innovation. The rapid growth of Vietnam's dairy sector has raised demands for higher milk quality, particularly in microbial control and food safety assurance.

Raw cow's milk serves as an ideal nutrient-rich environment for the survival and growth of various microorganisms. The microbial content in milk is a critical factor determining its quality, sensory characteristics, and the overall quality of dairy products [2]. Among the microorganisms present in milk, spore-forming bacteria are the most diverse and challenging to eliminate from the milk production chain, primarily due to their ability to transform into a dormant state - spores. Spores can survive under harsh environmental conditions, such as nutrient deficiency, osmotic pressure, and temperature fluctuations, owing to their multi-layered structure. While the outermost layer protects spores from enzymatic attacks, the inner layer maintains a dehydrated state and provides additional protection against chemicals. When favorable environmental conditions are restored, spores can germinate into their vegetative state [3].

The heat resistance of bacterial spores poses a major challenge in heat processing, especially since the enzymes they produce can cause undesirable changes in dairy products. Heat-stable enzymes, such as lipases and proteases, produced by Bacillus bacteria, could lead to undesirable biochemical changes that reduce the nutritional value and shelf life of dairy products [4]. Lipases catalyze the hydrolysis of fats, producing rancid odors and potentially reducing the viscosity and foaminess of milk [5]. Proteases degrade casein, resulting in bitter flavors and gel formation in milk [4].

To address this issue, heat treatment is considered the most effective method for controlling microorganisms and enzymes in milk, ensuring safety and extending the product's shelf life. Heat treatment can be divided into two groups based on temperature and objectives: pasteurization and sterilization. Pasteurization aims to inactivate the vegetative cells of pathogenic species present in food, extending shelf life but requiring the product to be stored under refrigeration. Sterilization is applied to ensure the stability of food products at room temperature, typically requiring

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temperatures above 100°C in most cases [6]. For milk and dairy products, sterilization is typically carried out at temperatures ranging from 110 to 135 °C for 10 to 30 minutes using in-bottle sterilization, or at 135 to 150 °C for 2 to 10 seconds using UHT processing, depending on the product type and the level of microbial contamination [7]. Sterilization can effectively deactivate microbial spores, enzymes, and toxins in food; however, it may significantly alter the sensory properties and nutritional composition of the product [6].

Therefore, the application of heat treatment requires careful consideration of temperature and time to avoid negative impacts on milk quality, especially when quantifying heat-resistant spores. Currently, various methods are used to quantify heat-resistant spores in dairy products. However, results from different methods cannot be directly compared due to differences in heat treatment procedures and culture conditions [8, 9]. According to the ISO (2009) [10] standard, the quantification of colony-forming units of heat-resistant spores of thermophilic bacteria in heat-treated milk and dried dairy products is performed using a colony-counting technique at 55°C after heating the sample at 106°C or 100°C for 30 minutes. However, in practice, heat treatment at 100°C is often preferred due to limitations in facilities and the reproducibility of higher temperatures. Different heat treatment methods and culture media are used to assess spore concentrations, but the selection of unsuitable media may lead to underestimation of spore concentrations due to limitations in spore germination and growth [9, 11]. Therefore, a global consensus on the most effective quantification method for heat-resistant spores is needed [8].

This study was conducted to evaluate the heat resistance ability in peptone and dairy products of *Bacillus* spp. strains isolated from cow's raw milk collected from smallholder farms under 100°C for heat treatment durations. The study will provide recommendations for local small-scale dairy producers to adopt appropriate heat treatment protocols, thereby improving product quality, saving energy, and minimizing environmental pollution.

MATERIALS AND METHODS

Materials

Eighteen samples of raw cow's milk were collected from milk collection stations at smallholder farms in the provinces of the Mekong Delta region, Vietnam. Each sample was randomly collected from milk tanks and stored in sterile plastic bottles in an icebox, then transported to the laboratory within a time frame of no more than 4 hours. Upon arrival, the samples were stored at 4°C until isolation procedures were carried out.

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Methods

Isolation, identification of heat-resistant spore-forming bacteria

Isolation: A 150 mL cow's raw milk sample was heat-treated at 80°C for 12 minutes in a water bath to kill vegetative cells, then immediately cooled in an ice bath [12]. A 1 mL aliquot of the diluted sample was spread onto Luria Bertani (LB) agar plates (containing, per liter: 10 g peptone, 5 g yeast extract, 10 g NaCl, and 20 g agar) and incubated at 37°C for 24 hours. The plates were observed after 24 hours of incubation; colonies exhibiting distinct morphology were selected and subcultured 3 to 4 times for isolation and purification to obtain pure bacterial isolates. The purified strains were stored in LB-glycerol medium (1:1) at 0°C for subsequent experiments [13]. The isolated bacterial strains were presumed to be heat-resistant spore-forming bacteria.

Identification: The purified bacterial strains were identified using Gram staining, motility testing, and catalase reaction methods following the guidelines of Bergey's Manual of Determinative Bacteriology [14]. The Gram-positive, motile, and catalase-positive strains were transferred into 50 mL of 1% peptone solution in sterile 250 mL screw-cap bottles and incubated at 37°C for 14 days to stimulate spore formation. After this period, the peptone solution containing bacteria was heat-treated at 80°C for 10 minutes to eliminate vegetative cells, yielding a spore suspension. The spore suspension was stored at 4°C for subsequent experiments [15].

Testing of the extracellular lipase and protease enzyme production

The ability to produce extracellular lipase and protease enzymes was determined following the method of Azman [16] by measuring the diameter of the hydrolysis zone (d, mm) around the colonies.

Determination of lipid-degrading enzyme activity: A 3 µL aliquot of the bacterial suspension was spotted onto Tween agar medium (containing, per liter: 10 g peptone, 5g NaCl, 10 mL Tween 20/Tween 80, 0.1g $CaCl_2.2H_2O$, and 20g agar). The plates were incubated at 37°C for 48 hours, and lipid degradation activity was evaluated by measuring the clear zone with fine particles around each colony [17].
Determination of protein-degrading enzyme activity: A 3 µL aliquot of the bacterial suspension was spotted onto LB agar supplemented with 1% (w/v) skim milk powder. The petri dishes were incubated at 37°C for 48 hours, and the presence of transparent zones around the colonies confirmed the ability of the strain to produce protein-degrading enzymes [18].

Heat-resistant spore ability testing

The efficacy of a sterilization method against a specific bacterial strain can be evaluated by measuring either the bacterial death rate or the survival curve, which is quantitatively expressed as the D_T value. The D_T represents the time (in minutes)

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required at a specific temperature to reduce the viable bacterial population by 90% [19].

The D_T value at a given temperature is calculated by the formula:

$$D_T = -\frac{t}{logN - logN_0}$$

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)

To determine the heat resistance of *Bacillus* spp. spores; 2.5 mL of the prepared spore suspension was transferred into a test tube (10 × 100 mm) and subjected to heat treatment using a temperature-controlled water bath. The heat treatment was conducted at 100°C for varying time intervals of 12, 15, 20, 25, and 30 minutes, respectively.

Following heat treatment, the spore suspension was immediately cooled in an ice bath to halt further thermal effects. Surviving spore enumeration was performed by transferring 1.0 mL of the heated spore suspension onto Luria Bertani (LB) agar plates. The plates were incubated at 37°C for 48 hours. After incubation, the number of colony-forming units (CFU) was determined using Plate Count Agar (PCA). The D_T value was calculated based on the reduction in spore counts, with each experiment conducted in triplicate to ensure accuracy; average results were derived from the three replicates [15, 20].

Heat-resistant spore survival in dairy products

Milk samples (unsweetened and sweetened milk) were randomly purchased from local stores, with their nutritional composition detailed in Table 1. Solutions (100 mL each) of the following were prepared: distilled water (control), peptone (10 g L^{-1}), unsweetened nutritional milk, and sweetened nutritional milk. These solutions were then sterilized at 121°C for 15 minutes.

To determine the heat resistance of spores in dairy products, 2.5 mL of the prepared spore suspension was transferred into separate containers, each containing 100 mL of one of the following: distilled water, peptone solution, sterilized unsweetened milk, and sterilized sweetened milk. The mixtures were thoroughly shaken and then subjected to heat treatment in a temperature-controlled water bath at 100°C for 30 minutes. After heat treatment, the samples were immediately cooled in an ice bath. Spore counts before and after heat treatment were determined using the Plate Count

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Agar (PCA) method. The experiment was repeated three times, and the average results were recorded [15, 21, 22].

Statistical Data Processing

The study results were analyzed using Microsoft Excel 2016. Statistical analysis was conducted using ANOVA at a 95% confidence level, followed by Tukey's test to compare and differentiate the means.

RESULTS AND DISCUSSION

Isolation results of heat-resistant spores in raw milk

A total of 30 pure bacterial strains were isolated from 18 raw cow's milk samples collected from cattle households in the Mekong Delta, Vietnam. All of them exhibited similar colony and cellular characteristics, including round or irregular colonies with rough surfaces, serrated or intact edges, opaque or ivory-white coloration, and a size range of 1–3 mm; microscopic analysis revealed short rod-shaped cells (Fig. 1); Gram-positive, motile, and catalase-positive.

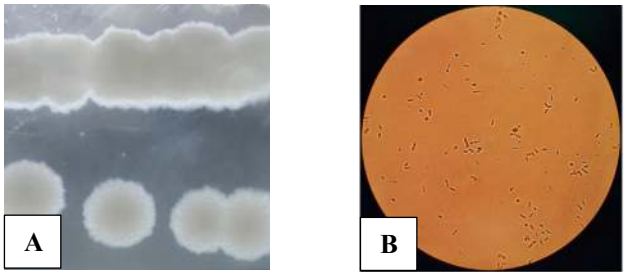

Figure 1: Colony (A) and cell (B) shapes of the isolated bacterial strain after 24 hours in LB agar medium

The prominent features morphological characteristics of the colonies and cells of the 30 isolated bacterial strains align with the typical features of heat-resistant spore-forming *Bacillus* spp., as described in Bergey's Manual of Determinative Bacteriology and supported by previous studies were reported by Huynh [23] in isolates from raw milk, and Li [24] in isolates from school canteen food.

Extracellular enzyme production ability of isolated thermotolerant spore-forming bacteria strains

Thirty strains of thermotolerant spore-forming *Bacillus* spp. were tested for the production of extracellular lipase and protease enzymes on various media after 48 hours of culture. Lipolytic ability was assessed by the appearance of a clear zone around the colonies, and proteolytic ability was assessed by the appearance of a transparent zone with higher transparency than the opaque white medium (Fig. 2).

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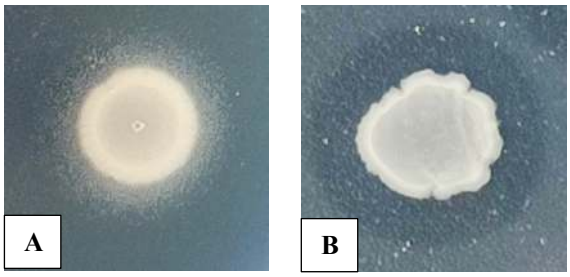

Figure 2: Lipid (A) and protein (B) degradation ability of isolated *Bacillus* spp. after 48 hours of culture

The results indicated that only 07 bacterial strains were capable of producing both types of extracellular enzymes (symbol: CT11.3; CT13.1; LA11.1; LA23.1; TG11.1; TG21.2, and TG33.2). Among these, strain LA23.1 exhibited the highest lipid-degrading ability (d=6.67±0.58 mm), while strain CT11.3 demonstrated the highest protein-degrading ability (d=13.33±0.58 mm), however, the differences were not statistically significant ($p > 0.05$) (Fig. 3).

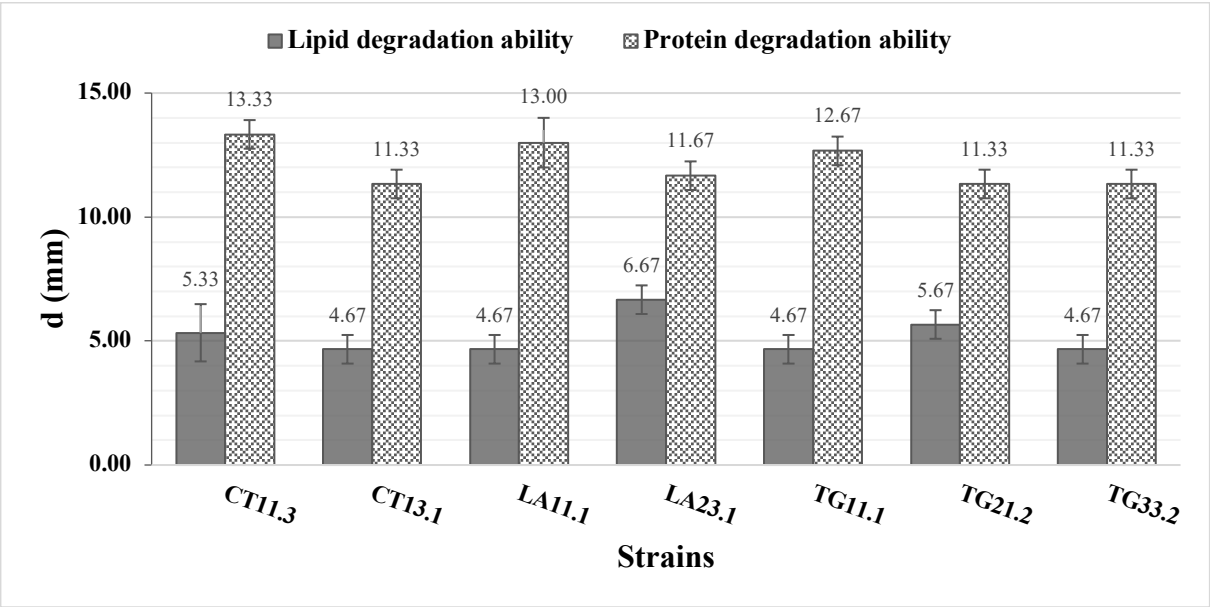

Figure 3: Extracellular enzyme production ability of 07 *Bacillus* spp. strains isolated after 48 hours of culture

According to the study by Chen [25], the presence of *Bacillus* spp. in raw milk and milk powder is the primary cause of milk fat degradation due to the production of lipase enzymes. These enzymes hydrolyze triglycerides, disrupt the physical properties of the milk emulsion system, and generate an unpleasant rancid odor, thereby reducing milk quality. Most lipase enzymes are produced by *Bacillus* spp. strains remain active even after pasteurization and UHT treatment. A recent study

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found that approximately 38% of *Bacillus* strains isolated from raw milk retained lipase activity after heat treatment at 142ºC for 4 seconds [26].

Bacillus spp. exhibit diverse proteolytic activity, and many species are capable of secreting more than one type of extracellular enzyme [27]. Protease is one of the primary extracellular enzymes associated with spoilage, causing putrefaction, off-flavors, bitter taste, and milk coagulation [18]. Many proteases are resistant to heat treatment and remain active during milk processing, even in the final sterilized product [28]. This highlights the need to control the risk of *Bacillus* contamination in milk to ensure product quality and safety.

In summary, seven bacterial strains (CT11.3; CT13.1; LA11.1; LA23.1; TG11.1; TG21.2 and TG33.2) have exhibited extracellular enzymes production abilities and were selected to test in next experiments.

Heat Resistance of Isolated Bacillus spp. spores

Evaluation of heat resistance in peptone medium of 07 bacterial strains producing 2 types of strong extracellular enzymes at 100°C for 12 minutes, 15 minutes, 20 minutes, 25 minutes, and 30 minutes. The results of decimal reduction time D_T are described in Table 2.

The results in Table 2 show that longer heat treatment durations reduce the heat resistance of *Bacillus* spp. strains, as evidenced by the decreasing D_T values with increasing heating time. For example, in strain **TG11.1**, when the heat treatment duration increased from 12 minutes to 15, 20, 25, and 30 minutes, the D_T declined from 8.72 minutes to 8.64, 8.56, 8.51, and 8.25 minutes, respectively.

Among a total of 07 bacterial strains, only the TG11.1 strain spores germinated after being heated at 100°C for 30 minutes, which was statistically significant ($p < 0.05$) compared to the other strains. In contrast, the spores of *Bacillus* spp. strains CT11.3, LA23.1 and TG21.2 were completely inactivated when treated at 100°C all time intervals tested. Meanwhile, the spores of the CT13.1 strain germinated after 25 minutes of heat treatment but were only completely inactivated after 30 minutes of exposure.

Fig. 4 shows the germination and growth of TG11.1 spores on agar medium at 37°C after 24 hours of incubation with heat treatment at 100°C after heating periods of 12 minutes, 15 minutes, 20 minutes, 25 minutes to 30 minutes.

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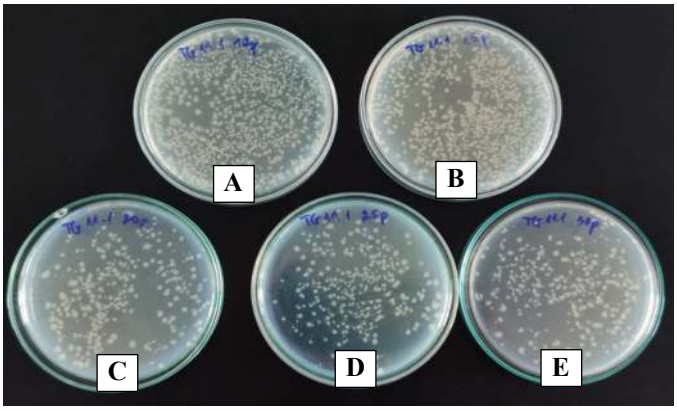

Figure 4: Spores of strain TG11.1 after heat treatment at 100°C for various times

A – 12 mins; B – 15 mins; C – 20 mins; D – 25 mins; E – 30 mins.

The study by Tatsinkou Fossi [29] reported the heat resistance of *Bacillus cereus* spores isolated from raw milk and dairy products in Cameroon, with D_{100} values ranging from 0.50 – 4.50 minutes. Janšťová and Lukášová [21] examined the heat resistance of 58 strains belonging to 9 *Bacillus* species isolated from raw milk and farm environments, finding an average D_{100} = 2.37 minutes. Additionally, Bui [22] isolated the *Bacillus cereus* strain OM1 from raw milk in Cần Thơ, Vietnam, with a D_{100} of 5.09 ± 0.17 minutes.

The destruction of spores by wet heat primarily occurs due to damage to core proteins and the denaturation of enzymes involved in metabolic processes. The wet heat resistance of spores is associated with the interaction between DNA and α/β-type small acid-soluble proteins (SASPs) in the nucleoid. Low core water content, combined with high levels of dipicolinic acid (DPA) and minerals, reduces molecular mobility within the core and protects proteins from thermal denaturation and aggregation [30].

Based on the results of this study, strain TG11.1 was identified as the most heat-resistant among the 07 strains selected to study heat resistance in dairy product media.

Heat resistance of *Bacillus* strain TG11.1 spores in dairy product

The spores of *Bacillus* spp. strain **TG11.1** were the only ones to survive the heat treatment at 100°C for 30 minutes in the peptone environment. Subsequently, these spores were tested for their heat resistance in dairy product at 100°C for 30 minutes. The D_T values are shown in Fig. 5.

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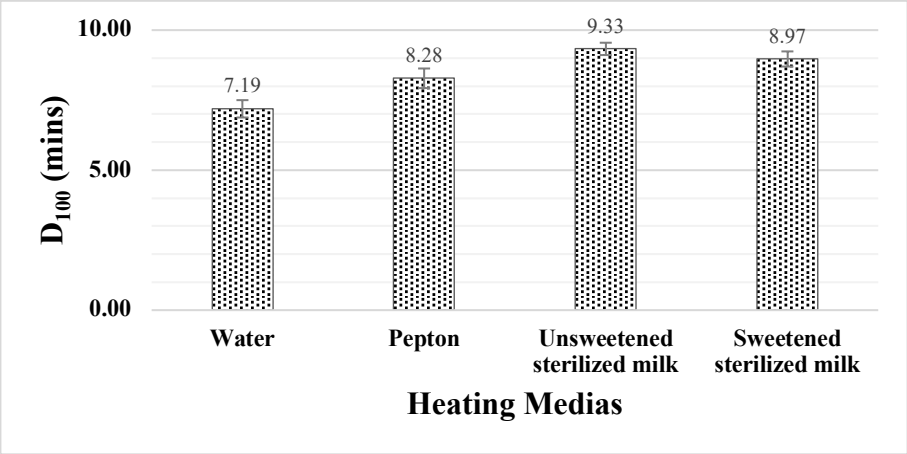

Figure 5: D_{100} of TG11.1 spores in different heating medias

The results in Fig.5 indicate that the heat resistance of TG11.1 spores in dairy product was significantly higher compared to distilled water and peptone media. The D_T corresponding to each environment (distilled water, peptone, unsweetened milk, and sweetened milk) was 7.19, 8.28, 9.33, and 8.97 minutes, respectively. The spores exhibited the highest heat resistance when treated in unsweetened milk (D_{100} = 9.33±0.22 mins), showing no statistically significant difference ($p > 0.05$) compared to the other media. In contrast, the lowest D_T was recorded in the distilled water environment (7.19±0.31 mins).

Huang [31] also concluded that the heat resistance of *Bacillus cereus* spores in a milk media was higher than in a Tryptic Soy Broth (TSB) culture medium when treated at 90°C for 5-20 minutes; however, no statistically significant difference ($p > 0.05$) was observed between the heat resistance levels across this time range in either media.

Several factors influence the heat resistance of microorganisms, one of which is the fat content in milk. A recent study found that fat helps protect bacteria from heat inactivation through two mechanisms: reducing the water activity of the cells and creating a moisture barrier that prevents water evaporation, thereby inhibiting inactivation [32]. The results of this study also align with these findings, as the D-values in the milk environment were observed to be higher than those in the peptone and distilled water media.

Additionally, the heat resistance of bacterial spores depends on the media in which they are heated. The maximum heat resistance of most microorganisms occurs when the water activity (a_w) is within the range of 0.20–0.40. Typically, in foods with water activity values (aw > 0.80), the heat resistance of microorganisms generally increases as water activity decreases. Some studies suggest that spores of *Bacillus cereus* exhibit high heat resistance in extended shelf-life (ESL) milk, with some even

surviving ultra-high temperature (UHT) treatment [33]. The increased heat resistance of spores is attributed to the lower water activity of the heating environment at high solid content [34], due to the high levels of lactose and mineral salts. However, the effect of water activity on spores or vegetative cells is complex, as specific solutes act as inhibitors; the presence of these solutes in the environment reduces the heat resistance of microorganisms. The discrepancy between increased water activity and the inhibitory effects of solutes may explain the conflicting data reported by various authors [35]. When evaluating the heat resistance of spores, it is important to consider that laboratory conditions cannot fully replicate the natural environment of spores. Therefore, differences in heat resistance compared to spores in their natural food environment must be taken into account [21].

Heat treatment is the most widely used processing technology in the dairy industry to reduce bacterial levels in milk. However, since spores can survive heat treatment, the most effective approach to reducing spore counts should be implemented at the farm level [3]. Additionally, heat treatment of milk for microbial control and enzyme inactivation affects its quality, nutritional value, and sensory properties. Therefore, optimizing the combination of temperature and processing time is crucial to extending product shelf life while minimizing quality degradation compared to raw milk [36].

To ensure microbial quality and consumer safety, the dairy industry must adhere to strict control measures and hygiene conditions, as recommended by guidelines for good dairy farming practices [37] and dairy plant hygiene standards ISO 8086:2004. Implementing Good Manufacturing Practices (GMP) and combining them with proper milk cooling at appropriate temperatures are effective measures for controlling thermophilic bacteria in milk. Cooling should begin immediately after milking, and the temperature should rapidly reach 4°C or lower to control microbial growth until heat treatment is applied [38].

CONCLUSION AND RECOMMENDATIONS FOR DEVELOPMENT

In this study, a total of 30 heat-resistant spore-forming bacteria was isolated from 18 samples of cow's raw milk collected from smallholder farms in the Mekong Delta, Vietnam. These strains exhibited morphological and biochemical characteristics typical of *Bacillus* spp. Among them, 07 strains demonstrated strong abilities to produce lipid- and protein-degrading enzymes. Notably, only the spore of TG11.1 strain could survive in a peptone medium after the pasteurization at 100°C for 30 minutes, with a decimal reduction time (D_T =8.54 mins) and exhibited greater heat resistance ability in dairy products (unsweetened and sweetened milk products) compared to peptone media and distilled water.

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The study recommends implementing strict hygiene protocols to minimize the contamination of cows's raw milk by *Bacillus* spp. Additionally, it proposes appropriate heat treatment regimes tailored to different dairy products for small-scale milk producers. These measures aim to reduce bacterial contamination during milk production, conserve energy, and extend the shelf life of dairy products.

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None

Conflict of interest

The authors declare no conflict of interest related to the manuscript.

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Table 1: Nutritional content of milk products

Nutritional content	Unsweetened milk	Sweetened milk
Fat	3.50 g	3.30 g
Protein	3.10 g	3.00 g
Carbohydrate	4.10 g	7.80 g
Calcium	110 mg	110 mg
Phosphorus	80 mg	80 mg
Selenium	7.50 mg	7.50 mg
Vitamin A	200 IU	200 IU
Vitamin D3	60 IU	60 IU

Note: Average nutritional composition per 100 mL.

Table 2: D_T value of 07 *Bacillus* spp. spores at 100°C in pepton media

Heating time (mins) \ Strain	CT11.3	CT13.1	LA11.1	LA23.1	TG11.1	TG21.2	TG33.2
12	0.00	6.07	2.86	0.00	8.72	0.00	2.69
15	0.00	5.86	2.72	0.00	8.64	0.00	2.63
20	0.00	5.80	0.00	0.00	8.56	0.00	0.00
25	0.00	5.42	0.00	0.00	8.51	0.00	0.00
30	0.00	0.00	0.00	0.00	8.25	0.00	0.00
Average	**0.00±0.00^{d}**	**4.63±0.09^{b}**	**1.12±0.07^{c}**	**0.00±0.00^{d}**	**8.54±0.14^{a}**	**0.00±0.00^{d}**	**1.06±0.05^{c}**

Note: The values were the average values of three repetitions. In the same row, values followed by a different letter represent statistically significant differences ($p<0.05$) by Tukey's multiple range test

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