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Research Article

Synergistic effects of organic acids with different solute concentrations on *Bacillus cereus* isolated from raw meat

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ABSTRACT

This study investigated the effects of combinations of citric and malic acids with different solutes (salt, glucose, and sucrose) on the survival of *Bacillus cereus* isolated from raw meat in laboratory broth. For the treatment of 0.5 and 1% organic acids (citric and malic acid), the addition of 3% solutes (salt, glucose, and sucrose) showed a synergistic effect, and also the purpose of this study was to assess the occurrence of *B. cereus* in raw meat (beef, mutton, chicken). *B. cereus* is gram positive facultatively anaerobic toxin-producing bacterium and usually found in nature and contaminates food. *B. cereus* is widely spread in food such as meat, and its products. A total of 60 samples of raw meats that include 20 beef, 20 mutton, and 20 chicken samples were taken from different local meat markets in Faisalabad and analyzed. 28 samples (46.6%) of raw meat out of 60 samples tested positive for *B. cereus*. The isolated pathogen was checked for organic acids treatment sensitivity. Results indicated that *B. cereus* was found more sensitive to citric acid than malic acid, but the addition of solutes increased the effectiveness of organic acids (malic and citric acids) which showed the synergistic activity of organic acid.

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Introduction

Meat is the edible portions of animals used for nourishment, including not only muscles and fat but also tendons and ligaments. Fresh meat has a short shelf life, which is a major challenge for global trade (Paredi et al. 2013; Boler and Woerner 2017). It is mainly composed of about 75% water, 19% protein, 1.2% carbohydrate, 2.5% fat, and 1.65% nitrogenous compounds. Meat is high in iron and vitamin B12 (Zhang et al. 2010; Beltrán and Bellés, 2019; Obeid et al. 2019). Meat can be categorized as red or white depending on the amount of myoglobin in the muscle fiber (Listrat et al. 2016). The pre-slaughter conditions of the animals can impact meat. Fresh meat is kept in the refrigerator, but it deteriorates due to facultative anaerobes and psychotropic aerobes. Packet meat and its byproducts can be deteriorated by psychotropic Lactobacillus (Hocquette et al. 2012). Depending on the hygienic circumstances in which meat is prepared, it may be exposed to a variety of infections that can be transmitted to humans (Abebe et al. 2020).

Food safety signifies the health of the general public, consumption of tainted food containing pathogen and infectious toxins produced by certain pathogens might cause serious illnesses (VanCauteren et al. 2017). These diseases have a major effect on public health and the epidemiology of these diseases is changing as recently recognized pathogens emerge and pathogens spread in prevalence (Altekruse et al. 1997). New pathogens can originate as a result of changing technology or ecology that links infections to the food chain, or as a result of virulence factors being transferred by bacteriophages (Tauxe 1997). The root causes include unhygienic methods of food production and preparation (Adley and Ryan 2016). Most foodborne infections are related to acute gastroenteritis as vomiting and diarrhea, but an affected person can also be ill with fever, abdominal cramps, and bloody stool (McCabe-Sellers and Beattie 2004). These conditions might be mild, requiring only a few days to cure, or severe, necessitating hospitalization and in some cases, death (Hoffmann and Scallan 2017). In less developed countries, foodborne infections constitute the major cause of sickness and death (Mead et al. 1999). These infections are a substantial global source of illness and mortality. In December 2015, World Health Organization (WHO) issued the first-ever estimates for foodborne diseases caused by a precise list of bacteria, parasites, and chemicals, subdivided by age, gender and region.

The range of foodborne pathogens involves a variety of aerobes, anaerobes and enteric bacteria, parasites, and viral pathogens. *Bacillus cereus* causes various severe foodborne illnesses in people around the world when they consume raw or contaminated food. *B. cereus* is a facultatively anaerobic gram-positive toxin-producing bacterium and is generally found in the natural environment and contaminates food. It can cause food intoxication, which frequently manifests as one of two symptoms: emetic or diarrheal (Gdoura et al. 2018). It can rapidly proliferate at room temperature and produce toxins. When ingested, these toxins can cause gastrointestinal (GI) disease (Nguyen and Tallent 2019). Because of variables which are favorable for bacterial survival and occurrence in production of food, *B. cereus* is detected in food. Poultry can become infected with *B. cereus* when growing out due to dusty housing conditions, contaminated chicks, or contaminated feed. Equipment and facilities in hatcheries have been polluted with cells that could infect chicks (Smith et al. 2004).

The nutritional requirements of the world concerning the accessibility of food changing that is not only nutritious, tasty, and free of pathogens but also free of toxic additives. Organic acids are fulfilling the demand for an antimicrobial preservative for food in process as food preservatives. To reduce the spoilage of food, key strategy was organic acids because the inhibitory effect of organic acids on infectious organisms growth has been used to preserve foods from spoilage and different acids vary significantly in their inhibitory effects (Hsiao and Siebert 1999). Acids are commonly utilized to preserve foods by being directly used in products or created due to fermentation process for enhancement of flavor (Mckellar and Knight 1999). Antimicrobial organic acids cross cell membranes and influence several cellular properties including ionic strength, intracellular environment, pH and acid concentration (Bjornsdottir et al. 2006), but organic acids usually found in foods differ in their structure and inhibitory effects for several bacteria (Nakai and Siebert 2003). Treatments of acids with salt and sucrose are commonly used to prevent microbial growth in a variety of acidified and fermented foods. When acetic, lactic, or propionic acids are treated with salt, antagonism is noticed and when malic acid, citric acid, or tartaric acid is treated with salt, a synergism is observed (Bae and Lee 2015). However, the relationship between various combinations of organic acids with solutes and the reduction of pathogens has not yet been fully expounded. Therefore, the effect of adding solutes (salt, glucose and sucrose) as food ingredient on the reduction of *B. cereus* in treatment with organic acids was examined in laboratory media in this study.

Materials and Methods

Collection of samples

In the months of April and May 2022, total 60 samples were collected from different local meat markets in the district Faisalabad, Pakistan. All raw meat samples were placed instantly into sterile plastic bags and were labelled. All the samples were transferred to refrigerator under aseptic conditions. The raw meat samples were taken to the cell culture research laboratory of Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan for further analysis.

Enrichment of samples

For enrichment of the collected samples, brain heart infusion (BHI) broth was used. 125ml of brain heart infusion broth was taken in sterile plastic bags with 25g of each raw meat sample and used stomacher to homogenize. Then these samples were placed in sterile tubes containing 5ml BHI broth and incubated at 37°C prior to culturing for enhancing the growth.

Streaking of samples on agar media

Directly onto nutritional media, plates were streaked a loopful of overnight cultures from BHI broth. Plates were incubated at 37°C for 24 hours. The petri plates were observed for significant development, 24 hours later. After 24 hours, colony morphology, Gram staining, and different biochemical analysis were used to presumptively identify *B. cereus*. After that colonies from nutrient agar plates were streaked on the Mannitol egg yolk polymyxin agar for *B. cereus* in order to obtain pure colonies.

Preparation of organic acid treatments

For this purpose, two commercially available citric and malic acids were used. The concentration of organic acids was 0.5 and 1%. These organic acids concentration was prepared by distilled water.

Effect of organic acids and different types of solutes

To evaluate the effect of solute with organic acids against *B. cereus*, 3% (w/v) solutions of salt, glucose, and sucrose were used. Tryptic soy broth containing 0.5 and 1% organic acids without solutes and with 3% salt, glucose, and sucrose were prepared. Pure bacterial culture (0.1mL) was inoculated into TSB (5mL). Samples were incubated at 25˚C and surviving cells were evaluated at 1, 2, 3, 4, and 5 days.

Enumeration of surviving cells

After treatments, to count the number of cells that were survived, samples were diluted 10-folds with serial dilution using 0.2% peptone liquid and diluent was spread-plated onto tryptic soy agar. Plates were incubated at 37°C for 24 hours and this process was repeated for next four days.

Statistical analysis

Colony-forming unit (CFU) was used to determine the inactivation of *B. cereus* cells. The CFU calculated by CFU = number of colonies x dilution factor / volume. Before analysis, the plate counts were converted to log10 CFU ml^{-1} for analysis of variance. Data were analyzed by ANOVA.

Results

Isolation and identification of *B. cereus* from raw meat samples

Isolation and identification were based on bacterial microscopic and macroscopic characteristics. Their identity was confirmed by biochemical testing. On nutrient agar, macroscopic examination revealed large granular, off-white-colored colonies with less wavy edge and less membranous consistency and typical colonies on MYP agar were pink in color. The isolated colonies were also examined using gram staining. They were visible under a microscope as gram-positive rods, short chain or single rods with square ends were found.

For the purpose of confirming the presence of *B. cereus*, biochemical tests such as the Voges Proskauer, citrate utilization, methyl red, indole, and catalase tests were also performed. Catalase, Voges Proskauer, citrate utilization, and methyl red tests all revealed positive results for *B. cereus* isolates and negative for indole test. Using sheep blood agar, a hemolysis test was performed, and Beta-hemolysis was seen in the clear space surrounding the bacterial growth as well as in rough, thick, dull grey colonies.

Percentage of positive isolates of *B. cereus* in raw meat

Sixty raw meat samples from local market were collected in sterile conditions from different shops, 28 raw meat samples were found positive for *B. cereus* and 32 samples were found negative. The occurrence of *B. cereus* was detected 47%.

Effect of organic acids and different types of solutes

According to a study, the effects of salt with organic acids were found to have either antagonistic or synergistic effects on the inactivation of Gram-negative bacteria in laboratory broth (Bae and Lee 2015) but in current study, organic acids showed synergistic effect against pathogenic microbes when different solutes were added in them. In current study, organic acids treatments showed that *B. cereus* is sensitive to citric and malic acids but survived malic and citric acids treatment. The initial count of *B. cereus* was approximately 10^7-10^8 $CFUml^{-1}$.

The amount of *B. cereus* decreased significantly with increasing time of treatments when treated with citric and malic acid alone, which was enumerated on tryptic soy agar. When treated with a combination of malic and citric acids with 3% solutes, greater reductions were seen in *B. cereus* which were enumerated on tryptic soy agar. Malic and citric acids, when combined with solutes, demonstrated synergism when compared to treatment with organic acid alone, it had much greater efficiency in killing the *B. cereus*.

Tubes that had salt were more effective against pathogen as compared to glucose and sucrose. 1% citric and malic acids with various solutes showed more effectiveness to kill *B. cereus* as compared to 0.5% malic and citric acids, but as incubation period increased 0.5% malic and citric acids with various solutes killed *B. cereus* more effectively. The results revealed that the survival of *B. cereus* in organic acids and different solutes was decreased more than for organic acids alone.

Enumeration of surviving cells

After treatments, to count the number of cells that were survived, samples were diluted 10-folds with serial dilution using 0.2% peptone liquid and diluent was spread-plated onto tryptic soy agar. Plates were incubated at 37˚C for 24 hours and this process was repeated for next four days.

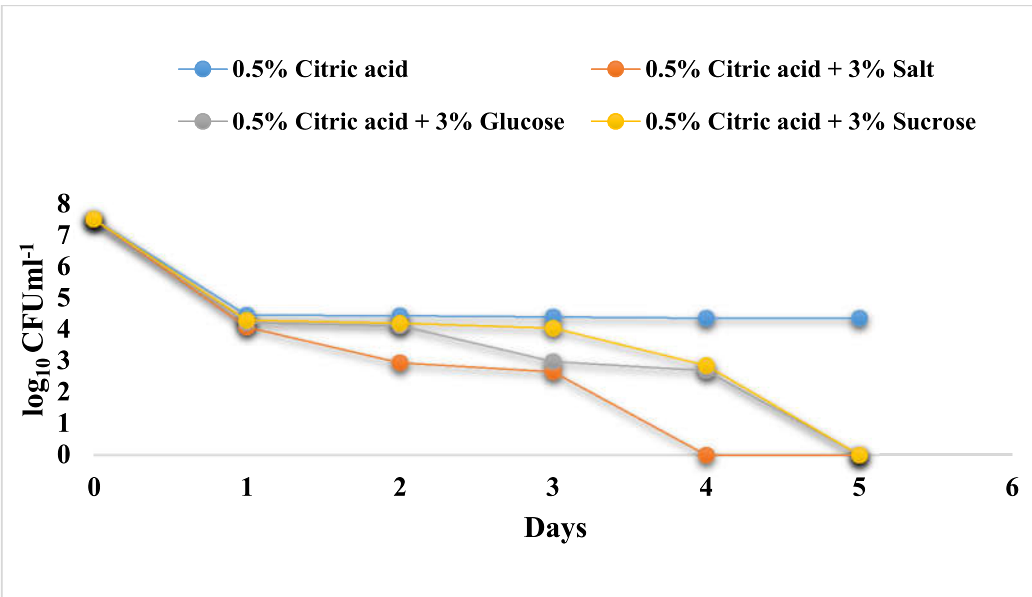
Discussion

Foods of animal sources, such as meat, milk, and eggs, are among the most significant sources of nutrients for humans because of their high protein and micronutrient levels. An important factor in the quality of its product can be the animal's hygiene before, during, and after slaughter. While being prepared and processed, meat and meat products may be contaminated with microbes. The contamination of meat is significant health concern (Dłubała et al. 2021). *B. cereus* causes foodborne infections in the restaurant and catering industries. The emetic and diarrheal intoxication syndromes, which result in foodborne illness, are typically self-limiting. Extreme intoxications, which frequently call for hospitalization and may even be fatal. Despite significant progress in recent decades in our understanding of *B. cereus* role in foodborne outbreaks, we still know very little about the gastrointestinal infections brought by *B. cereus*, which are likely related to infections acquired in hospitals (Deng et al. 2021). It is extremely alarming that *B. cereus* is becoming more common in foods of animal origin. Food derived from animals that has been contaminated by microbes may be carried antibiotic resistance (Cui et al. 2016).

Antimicrobial organic acids cross cell membranes and influence several cellular properties including ionic strength, intracellular environment, pH and acid concentration (Bjornsdottir et al. 2006). Acids treatment with salt and sucrose are generally utilized in fermented foodstuffs to prevent microbial growth (Bae et al. 2018). Aim of present research was to observe whether *B. cereus* was present in raw meat and to evaluate the synergistic effect of malic and citric acids with solutes against *B. cereus*.

In the current study, 60 raw meat samples (beef, mutton, chicken) were collected in aseptic conditions from different local meat markets of Faisalabad to investigate raw meat to evaluate the presence of *B. cereus*. The presence of *B. cereus* was reported 46.6%. Raw mutton meat was observed with high *B. cereus* incidence. The current study disagrees with (Tewari et al. 2015) in which they found *B. cereus* in raw meat was 29 out of 94 (30.85%). A study was done by Naas et al. (2018) in which they found the *B. cereus* in animal origin food was 39(29%). In the mutton samples used for this study, *B. cereus* was not found. However, in the present study, mutton samples were observed with *B. cereus*. Another study was done by GÜVEN et al. (2006) in which 22.4% samples were positive to contain *B. cereus*. In another study done by Rather et al. (2012), 37.45% raw meat samples were found positive. Another study that investigated the incidence of *B. cereus* in raw meat was carried out in China. Pathogen was found in raw meat, in this study, at a rate of 26.37% (Kong et al. 2021). The present study results were nearly agreed with Tharwat et al. (2020) who found *B. cereus* in 45% of

the meat and meat products. Another study(Solanki et al. 2019) showed the presence of *B. cereus* in 40 samples. Similar study was done by Smith et al. (2004) in which they found *B. cereus* in different marketed raw chicken meat. In their study, out of the 60 samples tested for this investigation, 27 samples had *B. cereus*. Another study was conducted by Hafiz et al. (2012) to evaluate the incidence of *B. cereus* from samples of mutton in the Kashmir valley during various seasons. *B. cereus* was found in 27 out of 60 mutton samples. Tharwat et al. (2020) also conducted a study to evaluate *B. cereus* in beef and beef products, 45% out of total samples were positive.
There have been several studies investigating the combination effect of acids and salt treatments against foodborne pathogens. Yoon et al. (2014) found



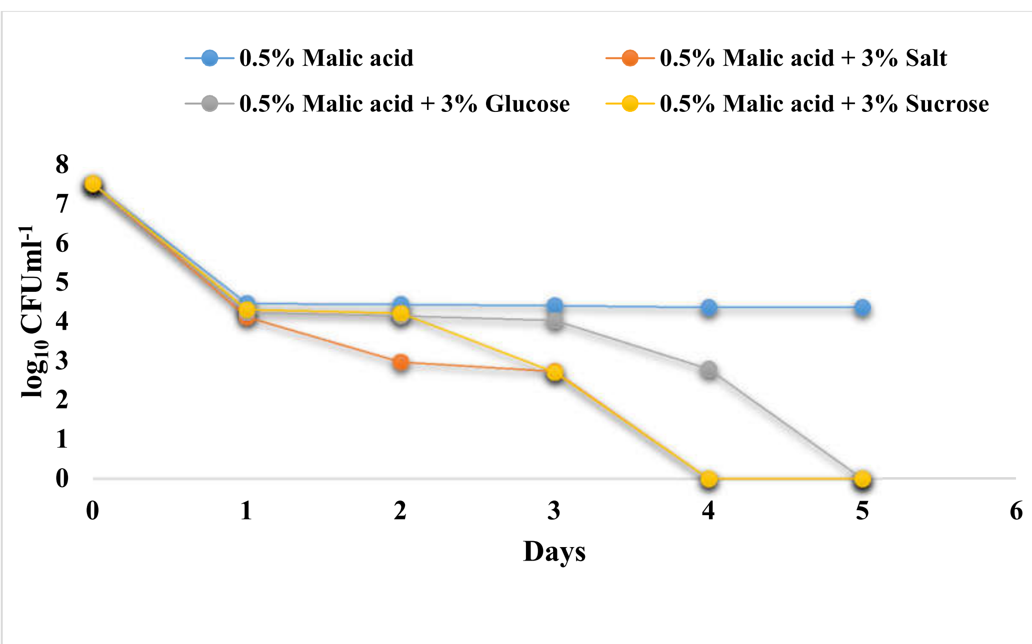
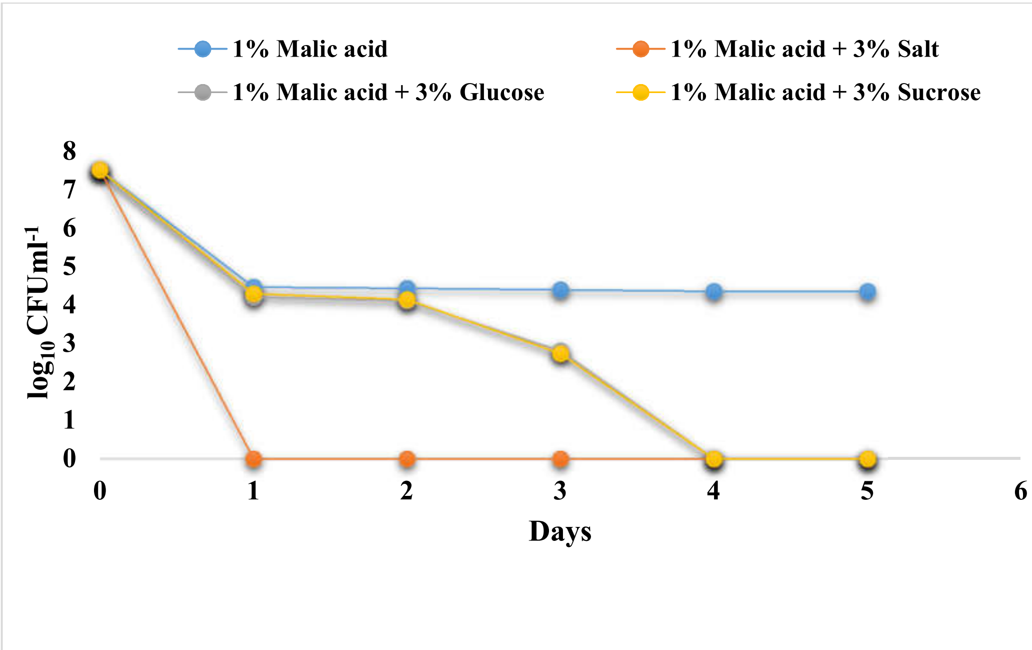


Fig. 1 & 2: Survival curves for *B. cereus* in tryptic soy broth without solutes with 3% salt, glucose, and sucrose treated with 0.5% organic acids (citric and malic acids). *B. cereus* was enumerated on tryptic soy agar plates



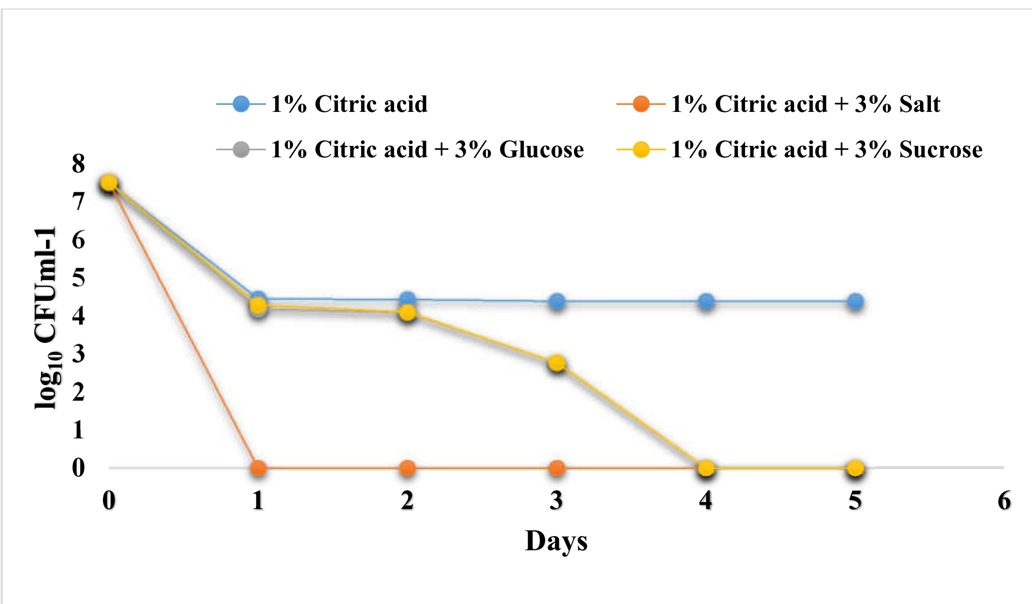
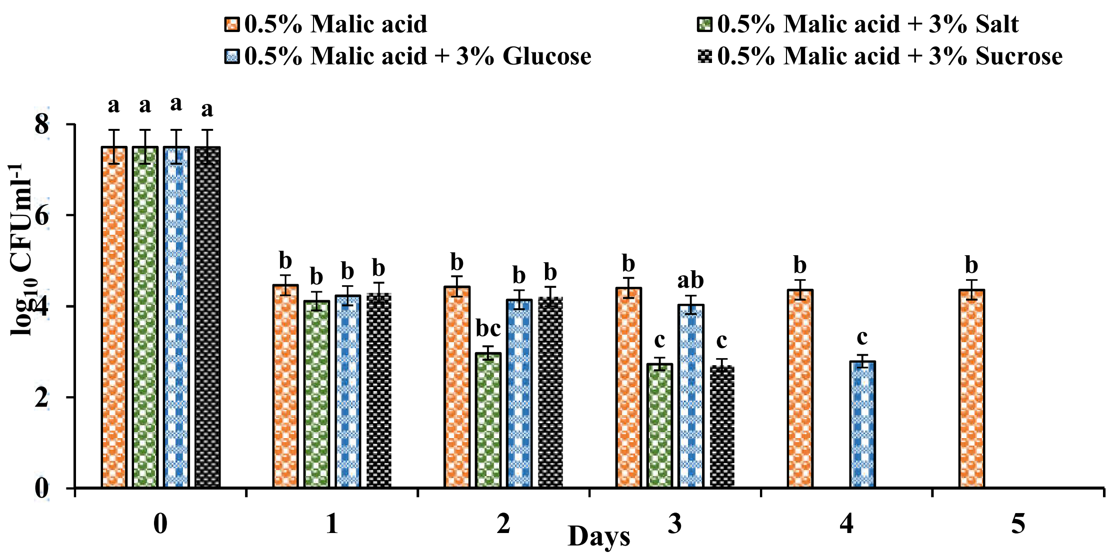


Fig. 3 & 4: Survival curves for *B. cereus* in tryptic soy broth without solutes with 3% salt, glucose, and sucrose treated with 1% organic acids (citric and malic acids). *B. cereus* was enumerated on tryptic soy agar plates



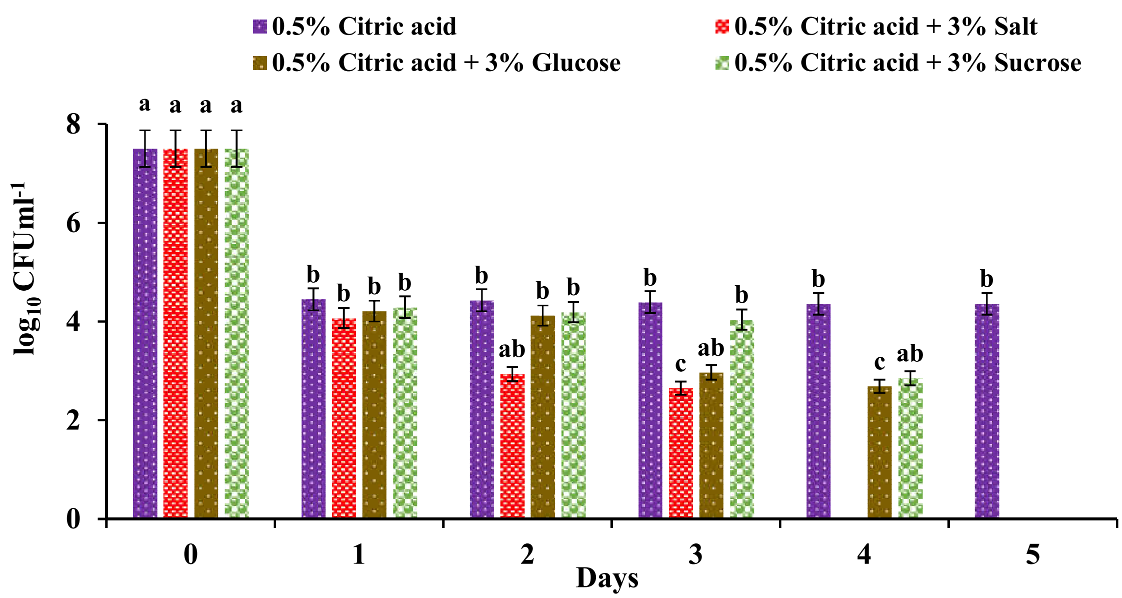
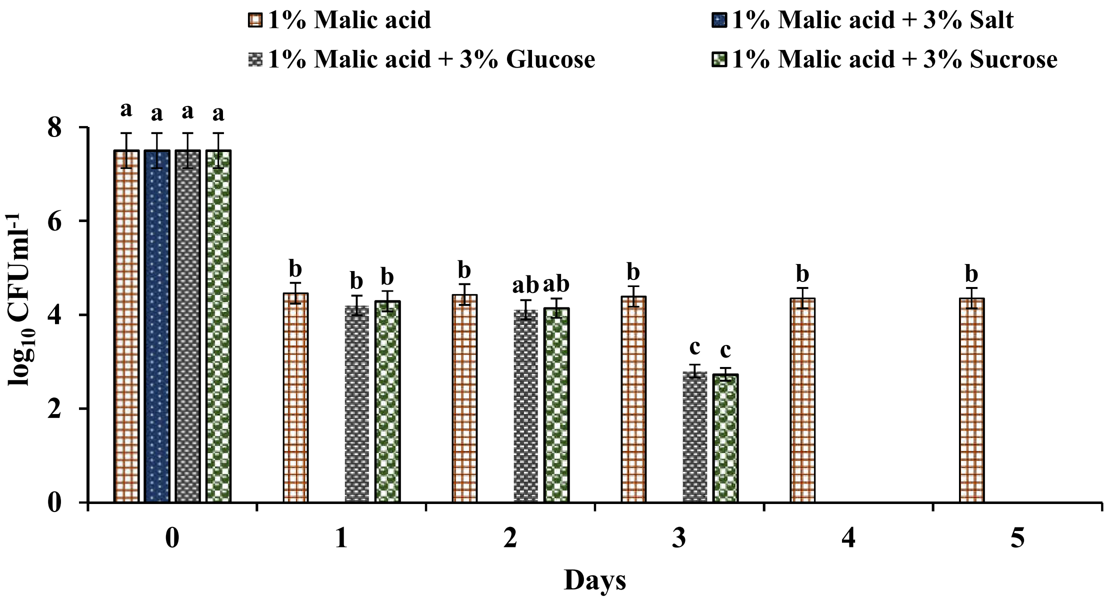


Fig. 5 & 6: Effect of different treatments of 0.5% citric and malic acids with different solutes (salt, glucose, and sucrose) against *B. cereus*
Each bar represents the mean of three replicates; means in the same column are not statistically different at a 5% level of significance



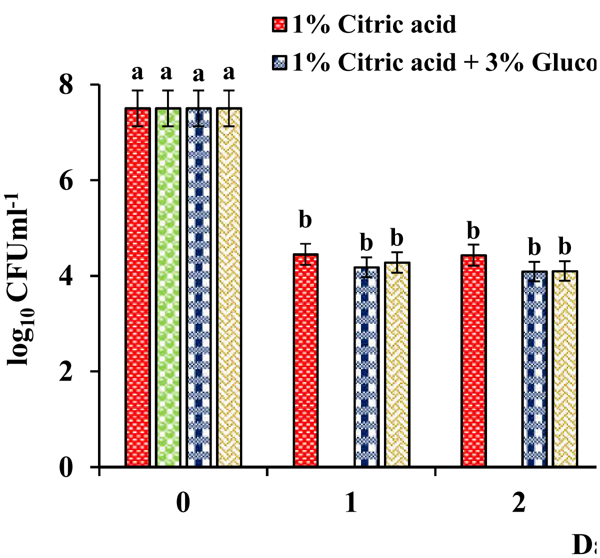


Fig. 7 & 8: Effect of different treatments of 1% citric and malic acids with different solutes (salt, glucose, and sucrose) against *B. cereus*
Each bar represents the mean of three replicates; means in the same column are not statistically different at a 5% level of significance.

that adding salt to treatments with citric and malic acid caused a significant decrease in the number of *Sh. flexnari*. According to Bjornsdottir et al. (2006), the protective impact of salt on *E. coli* O157:H7 survival rates in citric acid treatment was not seen. Stress generated by fatal acid (HCl, pH 3–5) or 14 % salt alone was less efficient than a combination treatment against *L. monocytogenes*(Shabala et al. 2008). According to Tiganitas et al. (2009), acid stress (lactic acid, pH 4–5) alone was less effective than the combination of acid and salt in reducing *L. monocytogenes*; however, acid and salt treatment was more resistant than acid treatment alone in reducing gram-negative bacteria like *E. coli* O157:H7 and *S. typhimurium*. Research revealed that some organic acids when combined with solutes showed antagonistic effect that supported the survival of pathogenic microbes in organic acids treatment indicated by Bae et al. (2021), but in current study, organic acids showed synergistic effect against pathogenic microbes when different solutes were added in them.

In current study, organic acids treatments showed that *B. cereus* is sensitive to citric and malic acids but survived malic and citric acids treatment. The initial count of *B. cereus* was approximately 10^7-10^8 CFUml^{-1}. The amount of *B. cereus* decreased significantly with increasing time of treatments when treated with citric and malic acid alone, which was enumerated on tryptic soy agar. When treated with a combination of malic and citric acids with 3% solutes, a greater reductions were seen in *B. cereus* which were enumerated on tryptic soy agar. Malic and citric acids, when combined with solutes, demonstrated synergism when compared to treatment with organic acid alone, it had much greater efficiency in killing the *B. cereus*. Tubes that had salt were more effective against pathogen as compared to glucose and sucrose. 1% citric and malic acids with various solutes showed more effectiveness to kill *B. cereus* as compared to 0.5% malic and citric acids, but as incubation period increased, 0.5% malic and citric acids with various solutes killed *B. cereus* more effectively.

Conclusions

The results of the present study revealed that the survival of *B. cereus* in organic acids (citric and malic acids) and different solutes (salt, glucose and sucrose) was decreased more than for organic acids alone. The order of synergistic effects of combined organic solutes (salt, glucose, and sucrose) and organic acids (citric and malic acids) against *B. cereus* was salt > glucose > sucrose.

These findings emphasize the significance of the combination of solutes (salt, glucose, and sucrose) with organic acids needed to lower the risk of pathogenic bacterial survival. The mechanism behind the synergistic effect of organic acids with solutes on *B. cereus* survival requires more research.

Competing Interest: The authors have no relevant financial or non financial interests to disclose.

Author Contribution: UH designed and SS performed the study; AB proof read the manuscript.

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