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FEASIBILITY OF USING SUCROSE LAURATE TO CONTROL THERMOPHILIC SPOILAGE IN LOW-ACID CANNED RATIONS

By
Anthony Sikes
and
Specialist Roy Flaig

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U.S. ARMY SOLDIER SYSTEMS COMMAND
NATICK RESEARCH, DEVELOPMENT AND ENGINEERING CENTER
NATICK, MASSACHUSETTS 01760-5020
SUSTAINABILITY DIRECTORATE

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Anthony Sikes
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13. ABSTRACT (Maximum 200 words)
The individual and combined effects of sucrose laurate (SL, L-1695), tert-butylated hydroxyanisole (B) ethylenediamine tetraacetate (E) and nisin were evaluated in a liquid growth medium (antibiotic assay medium + 1% soluble starch, AAMS) to determine their antagonistic action against the thermophilic flat-sour bacterium, *B. stearothermophilus*. Results indicated that on AAMS agar (pH 6.5 - 6.8) the minimum inhibitory concentration (MIC) of these additives required to prevent germination and outgrowth of *B. stearothermophilus* spores were > 50-60 ppm for SL and SLEB, while the MICs for BHA, EDTA and nisin were > 150, 350 and 400 ppm, respectively. During incubation (144 h at 55°C) in AAMS broth (pH 6.8) containing 50 ppm of SL and SLEB, respectively, the vegetative cell number increased ca. 2 $\log_{10}$ cycles, e.g., 10^3 -.10^5 CFU/mL. At similar concentrations of BHA, EDTA and nisin, no inhibition was observed. SL and SLEB did, however, prolong the lag phase and arrest log phase growth; BHA, EDTA and nisin did not exhibit similar inhibition tendencies. Differences in the inhibitory properties of SL and SLEB, relative to *B. stearothermophilus*, were not statistically significant ($p>0.05$). Under the conditions defined in this study, it would appear that SL and SLEB may have some utility in protecting thermally processed military rations from thermophilic spoilage.

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PREFACE

The results reported in this study represent the initial research efforts to assess the efficacy of sucrose esters as preservation adjuncts. The sucrose ester used in this study was sucrose laurate (SL). SL has been shown to be the most effective ester of the sucrose esters for controlling thermophilic spore activity. Although SL is an effective antimicrobial by itself, when combined with other GRAS food additives with antimicrobial activity, e.g., EDTA (E) and BHA (B), the overall antimicrobial activity of the combination is greater than any one of its components.

In this study, nisin, a GRAS food additive approved for use in pasteurized processed cheese to preclude the growth of *Clostridium botulinum*, will be compared to the antimicrobial effectiveness of SL and SLEB. The results from this investigation will be useful for assessing the feasibility of using these antimicrobials in low-acid canned, combat rations

This investigation was performed under the work unit titled "Attainment and Validation of Microbial Control", project # AH99BABOO, during the period of October 1990 through September 1993.

FEASIBILITY OF USING SUCROSE LAURATE TO CONTROL THERMOPHILIC SPOILAGE IN LOW-ACID CANNED RATIONS

INTRODUCTION

Complete thermal destruction of thermophilic spores in low-acid foods is difficult to impossible to accomplish without compromising product quality. The ideal situation would involve minimal thermal processing with enhanced product quality and the removal of the potential microbial activity of thermophilic spoilage in canned ration items, e.g., canned meats and vegetables. Currently, thermally processed foods in the ration system are heat processed in excess of what is required to achieve and maintain microbial stability. High heating of low-acid, canned rations is primarily done to eliminate the potential microbial activity of the foodborne pathogen, *Clostridium botulinum.*

As a consequence of the foregoing concerns, novel preservatives and combination preservation systems such as sucrose laurate (SL), ethylenediamine- tetraacetate (E), butylated hydroxyanisole (B) and the combination of SLEB, have been proposed that would address the potential thermophilic spoilage and excessive heating problems associated with low-acid canned rations. Previous research by Sikes and Whitfield (1992) and Kabara (1979 & 1981) have indicated the advantages of the combination method over more traditional food preservation methods.

Thus, the objective of the current investigation was to evaluate the antimicrobial effectiveness of sucrose laurate (SL), EDTA (E), BHA (B), and nisin alone and in combination, SLEB, on the germination and outgrowth of the thermophilic bacterium, *Bacillus stearothermophilus,* in a liquid growth medium.

MATERIALS AND METHODS

Spore preparation. A stock culture of Bacillus stearothermophilus spores (ATCC 12980), was obtained from the culture collection of the Microbiology Section, U.S. Army RD&E Center, Natick, MA. The stock culture was maintained on Cook and Brown sporulation Agar slants (Cook and Brown, 1964), stored at 1-4°C, and transferred monthly to maintain a viably active culture. Before each spore preparation, a loopful of the stock culture was transferred to freshly prepared Cook and Brown Agar plates and grown for 24 h at 55°C. The sporulation inoculum was prepared by scraping (bent glass rod) growth from the 24 h Agar plates and resuspending spores in phosphate buffer (pH 7.2; Schwab et al., 1984) to give spore density of approximately 10^7-10^8 spores/mL.

Prior to the inoculation of the sporulation medium, the spore suspension of *B. stearothermophilus* was activated by heating at 100°C for 15 min in a water bath heated with a Polystat 33 variable temperature immersion heater (BioBlock Scientific). To each Fernbach flask containing 500 mL of Cook and Brown media, three mL of the heated spore suspension was added and spread evenly over the surface of the sporulation medium.

All spore crops of B. stearothermophilus were prepared on Cook and Brown sporulation Agar according to the procedure described by Feeherry et al. (1987). After incubating for 4 d at 55°C, spores were harvested from Fernbach flask (cap. 2800 mL), washed 3x with phosphate buffer (pH 7.2; Schwab et al., 1984). To get rid of vegetative cells, the spores were resuspended in phosphate buffer (pH 7.2) containing 100 micrograms/mL of lysozyme and stirred at 37° C for 1 h. After enzyme treatment, spores were washed 4x with sterile deionized water to remove vegetative debris. Finally, spores were resuspended in 10 mL of sterile phosphate buffer (pH 7.2) and stored at 4°C until used. Two spore suspensions (crop) were prepared by the procedure described above.

Determination of thermal resistance. Using a Biological Indicator Evaluator Resistometer (BIER; Joslyn Valve Company, Macedon, NY), the decimal reduction times ($D_{121.1°C}$) were determined on aqueous suspensions of *B. stearothermophilus* spores (McCormick et al., 1988).

Using two independently prepared spore crops, 0.5 mL of heat-activated spores of *B. stearothermophilus* were placed in stainless steel cups (~ 3 mL capacity; inside diameter and height: 17 mm and 13 mm, respectively) and exposed to 121.1°C (250°F) for 0-20 min; survivors were determined at 5 minute intervals. At the completion of each heating cycle, the stainless steel cups were rapidly removed from the BIER chamber and submerged in sterile test tubes (25 x 150 mm) containing 9.5 mL of chilled phosphate buffer (4°C, pH 7.2). Samples remained chilled at 4°C until serially diluted. Dilutions were spread plated in duplicate (0.1 mL) on recovery Agar (antibiotic assay media supplemented with 0.1% soluble starch, AAMS; Cook and Brown, 1964). Plates were incubated aerobically for 24 h at 55°C. The resulting growth on AAMS represented the total viable count for a specific heating period (uninjured + injured).

Decimal reduction times ($D_{121.1°}$) were determined with a customized computer program (MS-DOS/GW-Basic, version 3.10, Wyse Technology, San Jose, CA) developed at Natick RD&E Ctr., Natick, MA. The program was written to facilitate the entry of experimental conditions, plate counts at each dilution and the sample volume plated. After data entry, the program takes the average of the $\log_{10}$ of the plate counts (duplicate plate counts/replication/time interval) and calculates the parameters of the least squares regression line and then takes the negative reciprocal of the slope of the regression line (D-value, min/90% decrease in survival).

Additives. Stock solutions of sucrose laurate (L-1695; Mitsubishi-Kasei America Inc., White Plains, NY) and EDTA (disodiumethylene-diamine-tetraacetate; Fisher Scientific Co., Fair Lawn, NJ) were prepared by suspending 1 g in 10 mL of distilled water (10 %, w/v).

BHA (2[3]-t-butyl-4-hydroxyanisole; Sigma Chemical Company, St. Louis, MO) was prepared by suspending 1 g in 10 mL of absolute ethyl alcohol (Quantum Chemical Corp., Tuscola, IL). After preparation, all samples were stored in tightly capped screw cap tubes at 1-4°C until used. Nisin was prepared according to the procedure outlined by Bell and Delacy (1987).

Minimum inhibitory concentrations (MIC). The procedure used to determine the minimum amount of SLEB, SL, BHA and nisin needed to inhibit germination/outgrowth of *B. stearothermophilus* spores was similar to the procedure described by Bargiota et al. (1987). Washed, heat activated spores of *B. stearothermophilus* were resuspended in PO_4 buffer, pH 7.2, and serially diluted through 10^{-8}. One-tenth mL portions of the 10^{-3} and 10^{-6} dilutions (ca. 10^2 and 10^5 spores/mL, respectively) were spread plated on AAMS Agar plates in triplicate. AAMS Agar plates containing each antimicrobial agent, SLEB (1:1:1, v/v), SL, EDTA, BHA, and nisin, were prepared by adding an appropriate volume of each stock solution (1000 ppm) to 200 mL of melted AAMS Agar and sterilizing at 121°C for 15 min. The concentration of each antagonist ranged from 50-400 ppm. The pH of the sterilized Agar ranged from pH 6.7-6.8.

Growth conditions. To duplicate 250 mL Erlenmyer flasks (Narcross, GA) containing 100 mL of growth media (AAMS broth) plus 1 mL of heat activated spore suspension of *B. stearothermophilus* (~10^3 to 10^4 spores/mL final concentration) the following samples were set up : control, no antimicrobials were added; SL, 50 ppm; EDTA, 50 ppm; SLEB, 50 ppm; BHA, 50 ppm; nisin, 50 ppm. Samples were incubated quiescently in a model 3916 Forma Scientific incubator (Marietta, OH) at 55°C for 144 h. Growth was monitored by removing 1 mL aliquots at 2 h intervals for the first 12 h and, two subsequent aliquots (1 mL)were taken at 24 and 144 h, serially diluted in 9 mL blanks of sterile phosphate buffer (pH 7.2) and surface plated(bent glass rod) on AAMS Agar plates. Inoculated plates were incubated at 55°C for 24 h before colonies were counted.

To determine the effects of adding SL and SLEB at some time other than 0 h on germination/outgrowth, duplicate flasks of 100 mL of AAMS broth were inoculated as described above but with some modifications in the antimicrobial additions (SL and SLEB). The following test protocol was set up: control, bacteria but no antimicrobial, SL-I, 100 ppm added at 0 h; SL-II, 100 ppm added at 3 or 4 h; SLEB-I, 100 ppm added at 0 h; SLEB-II, 100 ppm added at 3 or 4 h.

Statistical analysis. Differences in microbiological counts were examined for significance by analysis of variance using Statgraphics (Statistical Graphics Corp.,Rockville,MD). Data presented are the mean $\log_{10}$ values of two replications with duplicate subsamples. Significant differences ($p<0.05$) among treatment means were separated by Duncan's multiple range test.

RESULTS

The minimum inhibitory concentrations (MIC) of sucrose laurate (SL) EDTA (E), BHA (B) and SLEB required to prevent the germination ($D_{121.1^{o}C}$ 4-5 min) are shown in Fig. 1. Results indicated that in AAMS broth (pH 6.8) at 55°C, SLEB and SL (MICs ≥ 50 and 60 ppm, respectively) were more inhibitory, at a lower concentration, towards *B. stearothermophilus* than BHA, EDTA or nisin (MICs ≥150, 350 and 400 ppm, respectively). Based on the observed MIC values for SLEB and SL, no significant difference ($p > 0.05$) was shown to exist between the antagonistic effects of SLEB and SL against *B. stearothermophilus* spore germination.

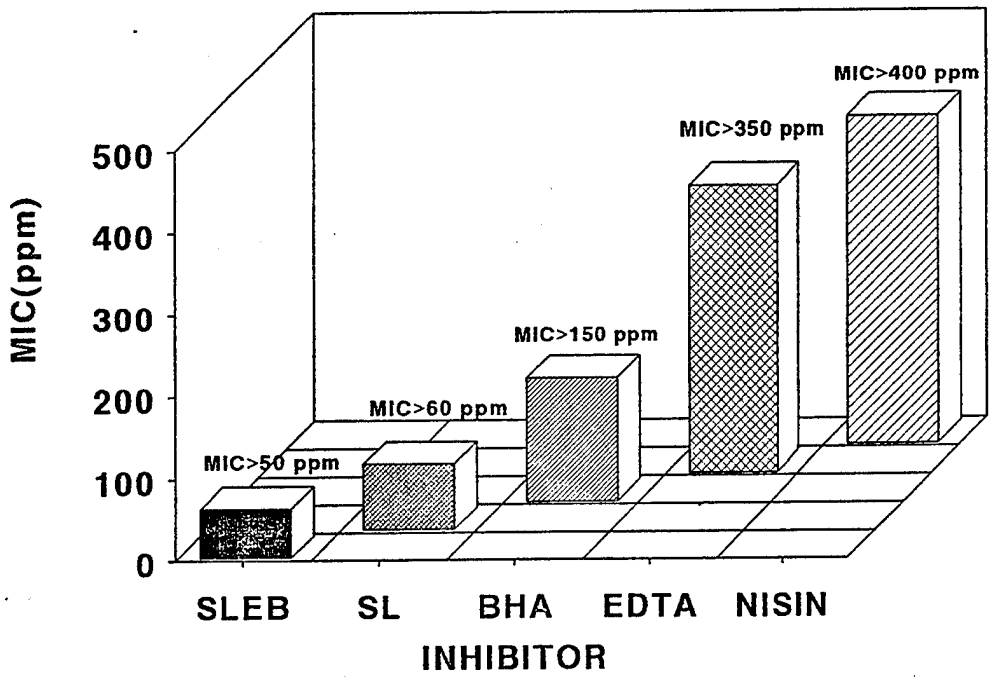

Figure 1. Minimum inhibitory concentration of sucrose laurate + EDTA + BHA (SLEB), sucrose laurate (SL), butylated hydroxyanisole (BHA) and nisin required to prevent germination and outgrowth of spores of *Bacillus stearothermophilus*

However, results from previous work with a nonsporeforming, gram-positive bacterium, *Listeria monocytogenes* (Sikes and Whitfield, 1992), revealed more inhibition to SLEB than SL. For example, in trypticase soy broth, 1000 ppm of SLEB totally inhibited the growth of *L. monocytogenes* during 12 d storage at 25°C, but, under the similar growth conditions, SL increased the lag period but was not inhibitory at 1000 ppm.

The germination and outgrowth of *B. stearothermophilus* in a liquid medium (AAMS broth, pH 6.8) containing 50 ppm of different antimicrobial agents were evaluated during a 6 d storage (55°C) period (Fig. 2).

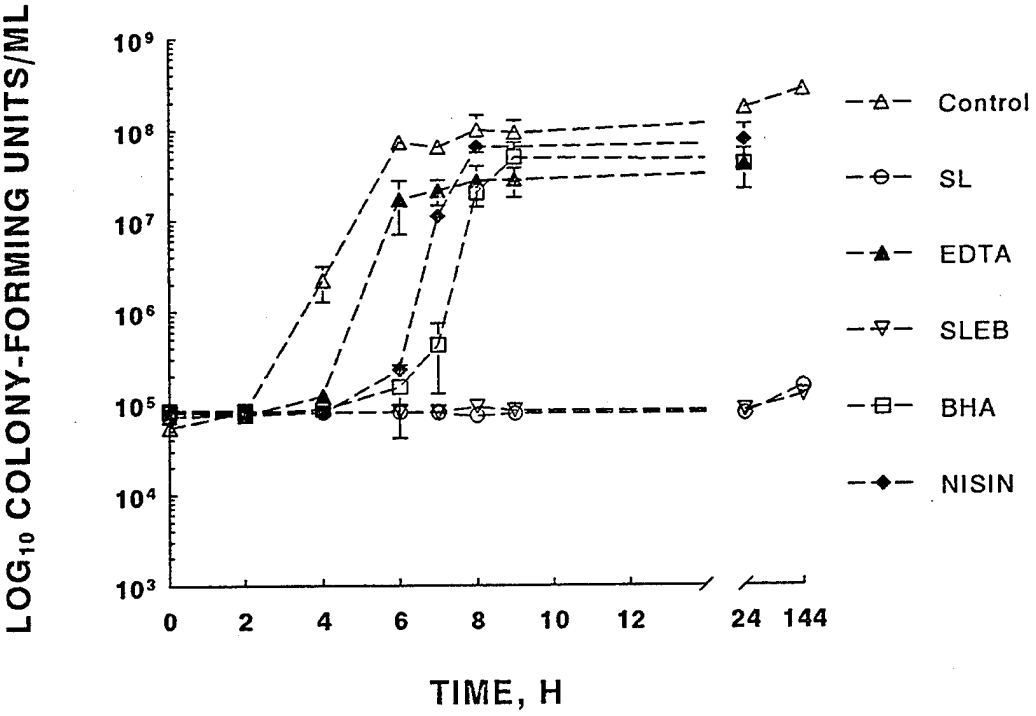

Figure 2. Inhibitory effect of SLEB, SL, EDTA, BHA and nisin on the germination and outgrowth of *B. stearothermophilus* spores in a liquid growth medium (AAMS, pH 6.8) at 55° C. All of the antimicrobial agents were used at a concentration of 50 ppm; the control samples contained only AAMS broth. Each point represents the grand mean of two experimental runs with two samples/run. Error bars represent the standard deviation of four experimental values.

At 50 ppm, all inhibitors except SL and SLEB were ineffective in precluding germination/ outgrowth of *B. stearothermophilus* spores; however, in the presence of EDTA (50 ppm), nisin (50 ppm) and BHA (50 ppm), the lag or onset of germination/ outgrowth of spores of *B. stearothermophilus* was increased. Maximum growth occurred in 10-12 h incubation at 55°C. SL and SLEB inhibited spore germination /outgrowth during the first 24 h of storage, with partial resolution of inhibition during the next 120 h of storage (< 0.5 $\log_{10}$ increase, Fig. 2). It was also demonstrated in this study that SL and SLEB were not only effective deterrents against spore germination but were equally effective at arresting log phase or vegetative growth of *B. stearthermophilus*. When 100 ppm each of SL and SLEB were added to broth cultures of *B. stearothermophilus* (AAMS, pH 6.8) at 0 h (SL-I and SLEB-I), growth was inhibited during 24 h of storage at 55°C (Fig. 3). If inhibitors (SL and SLEB) were added 3 h after inoculation into growth medium (AAMS), growth was immediately terminated (<1 h). After 3 h of growth, there was less than 1 log increase in the population; therefore, the rate at which growth decreased may be indicative of the spore germination/outgrowth stage.

However, when spores of *B. stearothermophilus* were allowed to germinate and grow out (55°C) for 4 h prior to adding the inhibitors (SL and SLEB), growth was arrested but less dramatically than at 3 h (Fig. 4). During the first 4 h of incubation (no inhibitor present), a 1-2 log increase in growth occurred. The amount of time required for the two inhibitors to terminate positive growth occurred over a much longer period. There may be several possible explanations for these apparent differences in the rates of growth: 1) after 3 h of incubation, germination/outgrowth was incomplete, e.g., a sizable proportion of the spore population remained in the dormant state, thus inhibition was apparently greater with this level of growth activity; 2) after 4 h of incubation, in which the cell population doubled several fold, the cell population was predominately vegetative ; 3) finally, SL and SLEB were apparently more inhibitory to bacterial spores than vegetative cells.

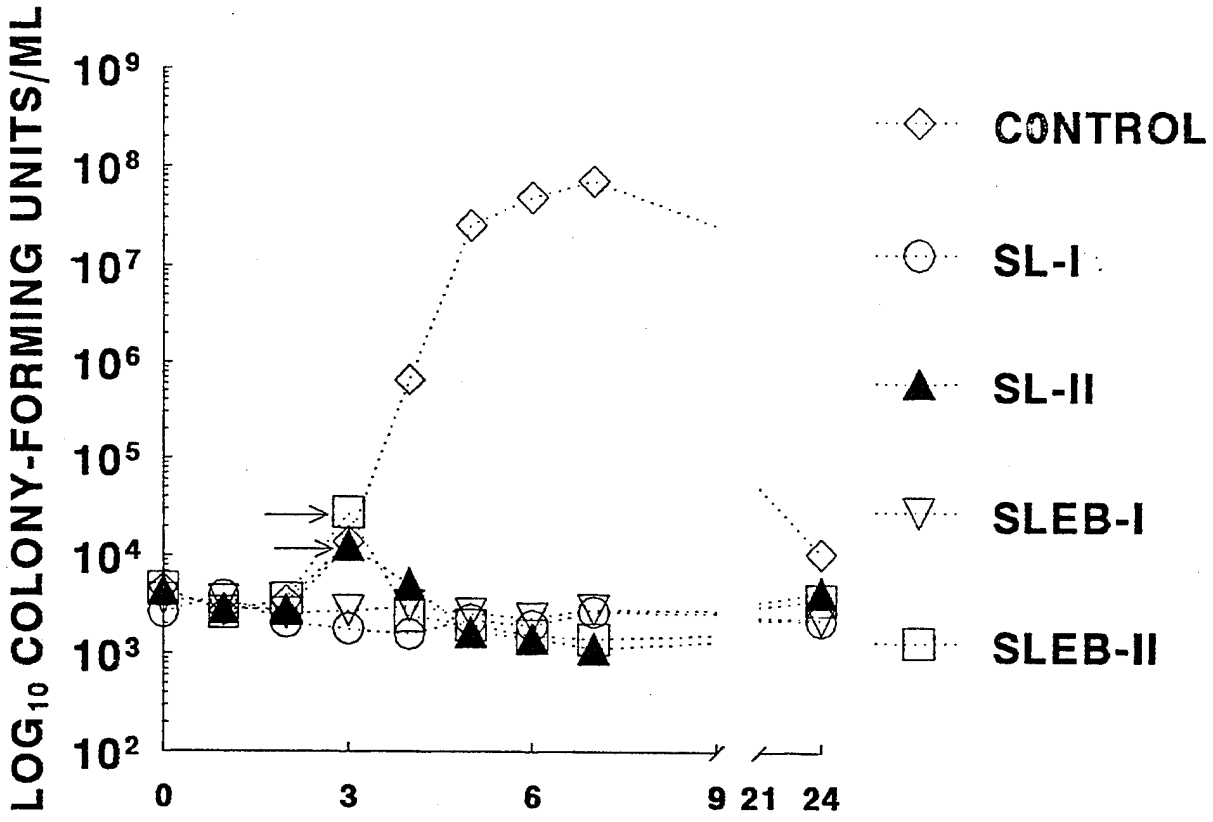

Figure 3. Comparative inhibitory effects of SL and SLEB on the germination and outgrowth of *B. stearothermophilus* spores when added at different times during the growth cycle (T_0: SL-I and SLEB-I, T_3: SL-II and SLEB-II; inhibitor level: 100 ppm). Arrows indicate the addition of the antimicrobial agents at T_3. Spores were cultured in AAMS broth (pH 6.8) at 55°C. Each point represents the grand mean of two experimental runs with two samples/run.

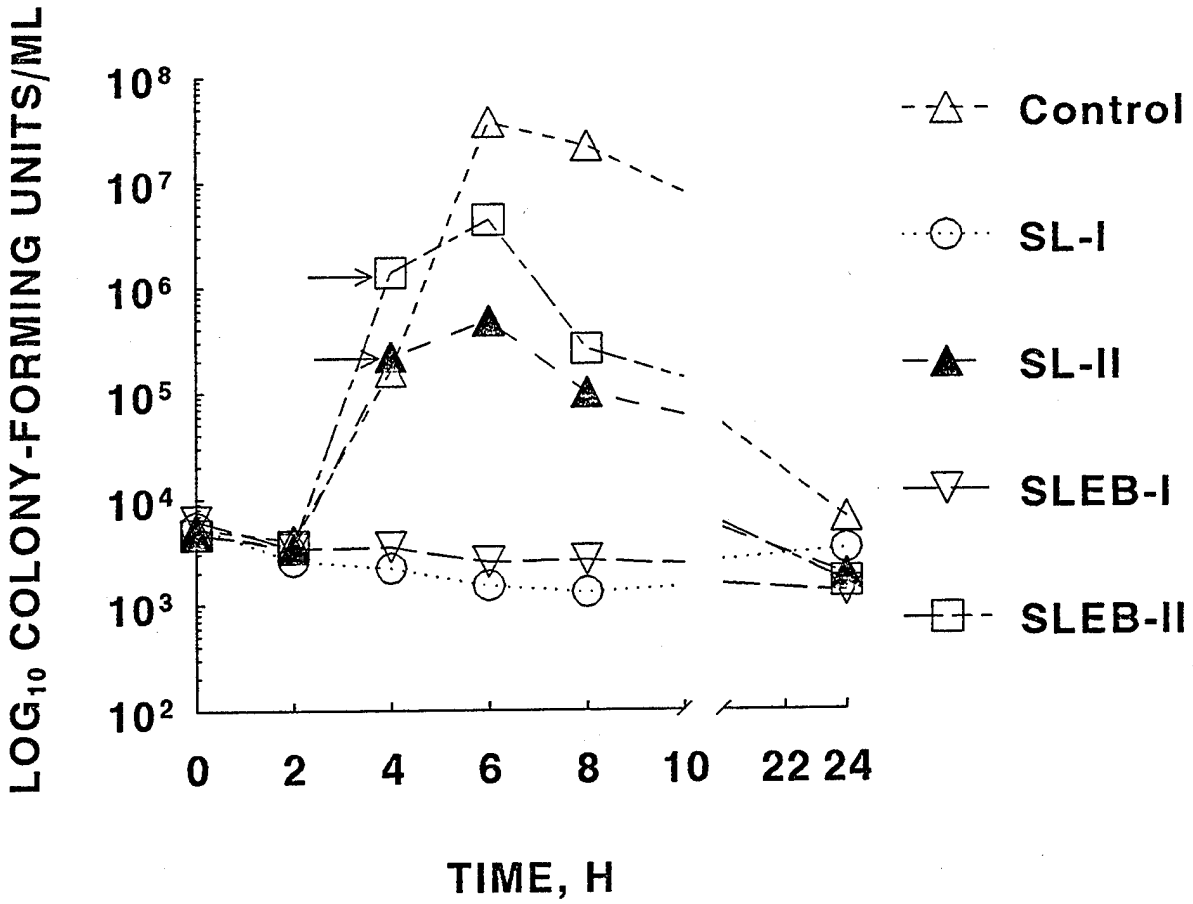

Figure 4. Comparative inhibitory effects of SL and SLEB on the germination and outgrowth of *B. stearothermophilus* spores when added at different times during the growth cycle (T_0: SL-1 and SLEB-1, T_4: SL-11 and SLEB-11; inhibitor level: 100 ppm). Arrows indicate the addition of the inhibitor at T_4. Spores were cultured in AAMS broth (pH 6.8) at 55° C. Each point represents the grand mean of two experimental runs with two samples/run.

CONCLUSIONS

The results indicated that sucrose laurate (SL) alone was very effective against thermophilic spore activity (e.g., germination and outgrowth). When SL was combined with GRAS substances , such as, ethylenediaminetetraacetate (EDTA), and butylated hydroxyanisole (BHA), there was no significant difference between the inhibitory effectiveness of SL and SLEB ($p > 0.05$). However, the results also showed that the antibacterial effect of SL and SLEB against spores of *B. stearothermophilus* was 2.5 to 5.1 times greater than GRAS compounds used alone. When compared to nisin, SL and SLEB are 6.7 times more inhibitory towards *B. stearothermophilus.*

It was demonstrated in this investigation that, in lab media, both SL and SLEB were effective antimicrobial agents against the germination/outgrowth of *B. stearothermophilus* spores. Individual components of the preservative system, SLEB, were usually less effective than the combined effects of all of the inhibitors. The exception was SL, which appeared to exert a comparable level of inhibition against the germination of *B. stearothermophilus* spores, when used alone. Nisin proved to be less of an effective antimicrobial against *B. stearothermophilus* than SL, BHA, SLEB or EDTA; however, the inhibition resulting from nisin and EDTA were very similar, e.g., MIC: 400 and 350 ppm, respectively

The results also imply that by using SLEB or SL the quality of thermally processed foods (low-acids foods) might be enhanced without compromising food safety or stability.

This document reports research undertaken at the U.S. Army Soldier Systems Command, Natick Research, Development and Engineering Center and has been assigned No. NRDEC/TR-96/26 in the series of reports approved for publication.

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