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Feasibility of using food-grade additives to control the growth of *Clostridium perfringens*

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Abstract

Previously, it was demonstrated that the combination of sucrose laurate (SL) ethylenediaminetetraacetate (E) and butylated hydroxyl anisole (B) (SLEB) was an effective antimicrobial agent against both gram-negative (aerobes) and gram-positive (facultative anaerobes) foodborne bacteria. This investigation examines the sensitivity of *Clostridium perfringens* to SLEB relative to: (1) the minimum inhibitory concentration (MIC) of SLEB required to inhibit the growth of *C. perfringens* and (2) the antibacterial effectiveness of different combination ratios of SLEB in fluid thioglycollate medium (FTM). Results indicated that the MIC of SLEB (1:1:1, v/v/v) against *C. perfringens* on tryptose sulfite cycloserine (TSC) agar was > 150 ppm at 37°C. However, in FTM, a SLEB (1:1:1, v/v/v) concentration of > 100 ppm inhibited *C. perfringens* during an incubation (anaerobic) period of 196 h at 37°C. The sensitivity of *C. perfringens* to different combination ratios was also investigated in FTM. The results showed that, when the concentrations of SL and E were held at 75 ppm in the SLEB combination, and the concentration of B increased from 0 to 75 ppm, *C. perfringens* growth increased initially during the first 24 h of incubation (37°C) but remained constant during the next 48 h. Similarly, when concentrations of SL and E were held constant at 150 ppm in the SLEB combination and the B ratio increased from 50 to 150 ppm in FTM, *C. perfringens* viability decreased in all of the treated samples during 72-h incubation at 37°C. The results indicated that SLEB was an effective inhibitor of *C. perfringens* growth activities, and the ratios of the components of SLEB can be adjusted to meet specific preservation needs. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: *C. perfringens*; Antimicrobial agent; Sucrose laurate; BHA; EDTA

1. Introduction

Sucrose fatty acid esters are Generally Recognized As Safe (GRAS) multi-functional food additives that have regulatory approval as stabilizers and emulsify-

ing agents in baked goods, dairy products, and as components of protective coatings applied to fresh fruits in the United States. In addition to their primary function in food as emulsifiers, sucrose esters have been shown to control thermophilic spore activity in hermetically packaged foods (Kabara, 1982; Nakayama et al., 1982; Suwa et al., 1986; Tsuchido et al., 1987). Although the mechanism of

spore inhibition by sucrose fatty acid esters is not well understood, evidence presented in this study as well as other investigations clearly show that both mesophilic and thermophilic spore-forming bacteria are particularly sensitive to the presence of sucrose laurate (Sikes, 1995; Sikes and Whitfield, 1992), especially when in combination with other multifunctional food additives, such as ethylenediaminetetraacetate (E), a chelating agent, and/or butylated hydroxy-anisole (B), a phenolic antioxidant.

A comparison of the minimum inhibitory concentration (MIC) of combinations of sucrose laurate (SL) + ethylenediaminetetraacetate (E) + butylated hydroxyanisole (B) required to prevent growth of several foodborne bacteria was reported by Sikes and Whitfield (1992) and Sikes (1995). The results indicated that the spore-formers, *Bacillus stearothermophilus* and *Bacillus coagulans*, were 2–7 times more sensitive to SLEB (1:1:1, v/v/v) than the nonspore-forming pathogenic bacteria, *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella typhimurium*. The role that E and B play in microbial inhibition is better understood. According to Kabara (1979), E has minimal antimicrobial activity but functions by enhancing the germicidal activity of the lipophilic substances, such as sucrose esters and phenolic antioxidants, perhaps by competing with the cell membrane for stabilizing calcium ions. The mechanism of inhibition of the phenolic antioxidant, B, is believed to be related to its ability to disrupt the functions of the cell, such as synthesis of nucleic acids, proteins and lipids, and functions of the mitochondrion (Raccach, 1984).

Although limited information is available on the ability of sucrose esters to control pathogenic (anaerobic) spore-formers in food, Ando et al. (1983) found that when 100–400 ppm of sucrose myristate (M-1695) were added to chicken soup inoculated with *C. perfringens* spores (NCTC 8238, type A), no growth occurred and the number of viable cells decreased at 37°C. Their findings also showed that 100–1000 ppm of M-1695 prevented the germination of spores *C. botulinum* (types 62A and E Iwanai) in thiotone medium (37°C).

C. perfringens is an important anaerobic, gram-positive spore-forming rod that produces a foodborne illness when foods containing a large population of spores or vegetative cells, e.g., $>10^5$ cfu/g, are ingested (Shandera et al., 1983). Work by Klin-

dworth et al. (1979) showed how effective B was against *C. perfringens*. These researchers found that in fluid thioglycollate medium (FTM), B at 200 ppm was bactericidal against several *C. perfringens* strains; B was also found to act synergistically when used in conjunction with other food antimicrobials, i.e., nitrite, sorbic acid and parabens.

Based on data generated from previous studies (Sikes, 1995; Sikes and Whitfield, 1992; Sikes and Flaig, 1996), *C. perfringens* has been shown to be the least sensitive spore-forming bacterium to SLEB. Therefore, the objective of this investigation was to determine the feasibility of using sucrose laurate in combination with E and B to prevent the growth of the mesophilic anaerobe *C. perfringens* in laboratory media. The advantage of a combination approach to food preservation is that presently approved multifunctional food additives, which are either approved for food use or have GRAS can be used to design preservation systems that will meet specific microbiological requirements (Kabara, 1981).

2. Materials and methods

2.1. Spore preparation

A stock freeze-dried culture of *Clostridium perfringens* (ATCC 12916) was revived according to the procedures outlined in the American Type Culture Collection Catalogue (Ghera et al., 1985). After opening the sealed vial, the freeze-dried material (cells) was placed in approximately 5 ml of sterile brain heart infusion broth (BHI; Difco, Detroit, MI) and allowed to set several hours before inoculating (1 ml) several tubes of liver broth (15 ml; Schwab et al., 1985) with the BHI suspension. The liver broth (LB) cultures were incubated anaerobically (90% N₂–10% CO₂, GasPak Anaerobic System, BBL) for 20–24 h at 37°C. The next day a suitable aliquot (0.1 ml) of the active LB culture was placed in several tubes of fluid thioglycollate (FTG) broth (10 ml; BBL) grown anaerobically (GasPak Anaerobic System, BBL) overnight at 37°C before inoculating sporulation media. Spore production followed the procedures described by Duncan et al. (1972). Ten ml of the active FTG broth culture were inoculated into 100 ml of DS sporulation medium (Duncan and Strong, 1968) and incubated at 37°C for 3 h. Then

the 100 ml of DS sporulation medium was added to 1000 ml of fresh DS sporulation medium and incubated (37°C) for 24 h. Finally, the sporulation frequency was determined on the 24-h culture.

The colony-forming units in the DS sporulation medium (V) was determined by serially diluting the culture in phosphate buffer (NaH_2PO_4 – Na_2HPO_4 , 0.05 M, pH 7.0) and plating on to tryptose–sulfite–cycloserine (TSC) agar (Difco) without cycloserine. The heat-resistant spore population (S) was determined by heating an aliquot of the culture at 75°C for 20 min and plating on to TSC agar without cycloserine. Plates were incubated anaerobically for 24 h at 37°C (GasPak Anaerobic System, BBL). The percent spore viability was measured as the ratio of S/V . The final *C. perfringens* spore crop (ca. 32×10^7 viable spores per ml) was washed and centrifuged ($3000 \times g$) three times in 0.05 M sodium phosphate buffer (PO_4), pH 7.0, and stored in 0.05 M PO_4 buffer (pH 7.0) at 1–5°C until used.

2.2. Additives

Sucrose laurate (SL, L-1695) was a gift from Mitsubishi-Kasei Food, Tokyo, Japan. The purity of the combined fatty acid was 95%; however, the ester composition was 80% monoester and 20% di-, tri and polyesters. Disodium ethylene-diaminetetraacetate (E. Fisher Scientific, Fair Lawn, NJ) and SL were dissolved in deionized water. Butylated hydroxyanisole (B; 2[3]-*t*-Butyl-4-hydroxyanisole, Sigma Chemical, St. Louis, MO) was prepared by dissolving in absolute ethyl alcohol (Quantum Chemical, Tuscola, IL). After preparation, all stock solutions were filter sterilized and stored (1–5°C) in screw-cap tubes tightly sealed and wrapped with parafilm (American National Can, Greenwich, CT) until used. Before each experiment, an appropriate quantity of each additive was added to media (FTM or TSC agar) and autoclaved at 121°C (15 psi) for 15 min.

2.3. Minimum inhibitory concentration (MIC)

The method used to determine the minimum amount of SLEB required to inhibit growth of *C. perfringens* was similar to the procedures described by Bargiota et al. (1987). An aliquot of washed and resuspended (PO_4 buffer, pH 7.0), spores of *C.*

perfringens was removed from the stock suspension, heat activated [20 min at 75°C in a water bath (GCA Precision Scientific, Chicago, IL)] and serially diluted in PO_4 buffer (pH 7.0) to give final spore densities of ca. 10^3 and 10^6 spores per ml. Subsequently, 0.1-ml aliquots of each spore dilution were spread plated with a bent glass rod on TSC agar plates containing various concentrations of SLEB (1:1:1, v/v/v). Inoculated plates were incubated anaerobically for 48–72 h at 37°C. The lowest concentration of SLEB that prevented growth was considered the minimum inhibitory concentration (MIC). Plates were done in triplicate.

TSC plates containing various concentrations of SLEB (1:1:1, v/v/v) were prepared by adding an appropriate volume of each stock solution to 100 ml of nonsterile, molten agar and sterilizing at 121°C (15 psi) for 15 min. TSC plates without SLEB were used as controls.

When growth was monitored by optical density (O.D.), a protocol similar to the one described above was followed. At each sampling interval, approximately 5 ml of sample was aseptically removed from the reaction flask, placed in 10-mm Spectronic 21 cells (Milton Roy, Rochester, NY) and the absorbance was read at 540 nm.

2.4. Statistical analysis

Differences in \log_{10} microbiological counts were examined for significance by one-way analysis of variance and Duncan's multiple range comparisons (Minitab™ Statistical Software, Minitab, State College, PA). Data presented are the mean \log_{10} colony-forming units of two or three replications with two or three samples per rep.

3. Results

The MIC of SLEB (1:1:1, v/v/v) required to inhibit *C. perfringens* (ATCC 12916) growth on TSC agar plates was >150 ppm. However, when a similar SLEB combination ratio was examined in fluid thioglycollate medium (FTM), a lower concentration was required to effect the same level of inhibition. For example, at 50 ppm of SLEB (1:1:1, v/v/v), *C. perfringens* growth activities were not

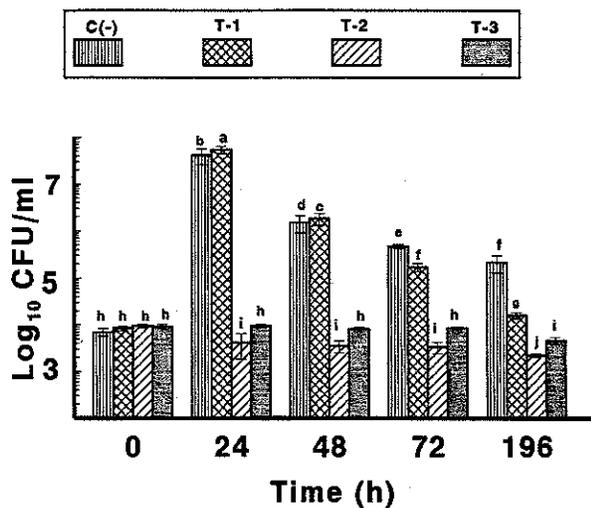


Fig. 1. Inhibitory effect of SLEB on the growth of spores of *C. perfringens* in fluid thioglycollate medium (pH 6.8) at 37°C. FTM was inoculated with 10^3 – 10^4 spores per ml at time 0. Cell counts were enumerated on TSC agar (24–48 h at 37°C). SLEB (1:1:1, v/v/v) treatments were C(-), no SLEB; T-1, 50 ppm SLEB; T-2, 100 ppm SLEB; T-3, 200 ppm SLEB. Bar heights represent the mean of two independent experiments with three subsamples. Means with the same letter superscript are not significantly different ($P < 0.05$). Error bars indicate the standard deviation, $n = 6$.

affected, but at > 100 ppm growth was arrested (Fig. 1).

The sensitivity of *C. perfringens* to different SLEB combination ratios was also examined in FTM (Figs. 2 and 3). After the initial 24 h of incubation (37°C) in FTM, *C. perfringens* growth, as measured by changes in absorbance (A_{540}) in the SLEB-free control (S-0), increased to a mean O.D. (A_{540}) value of 0.57 (Fig. 2). During the next 48 h of incubation, the cell density increased to an average O.D. value of 0.65 (Fig. 2). Concurrently, four SLEB combination ratios were evaluated at 75 ppm for SL and E: S-1 (1:1:0, v/v/v), S-2 (1:1:0.33, v/v/v), S-3 (1:1:0.66, v/v/v) and S-4 (1:1:1, v/v/v). Butylated hydroxyanisole (B) was incrementally increased from 0 in the combination ratios to 75 ppm (Fig. 2). After the initial 24-h incubation (37°C) period, S-1, S-2, S-3 and S-4 treatments resulted in mean O.D. (A_{540}) increases in cell density of 0.34, 0.42, 0.42 and 0.16, respectively (Fig. 2). Over the next 48 h of incubation at 37°C (24 through 72 h), growth (A_{540}) in the control increased significantly ($P < 0.05$), while there was no significant change in growth (A_{540}) of the treated samples ($P < 0.05$; Fig. 2). Among

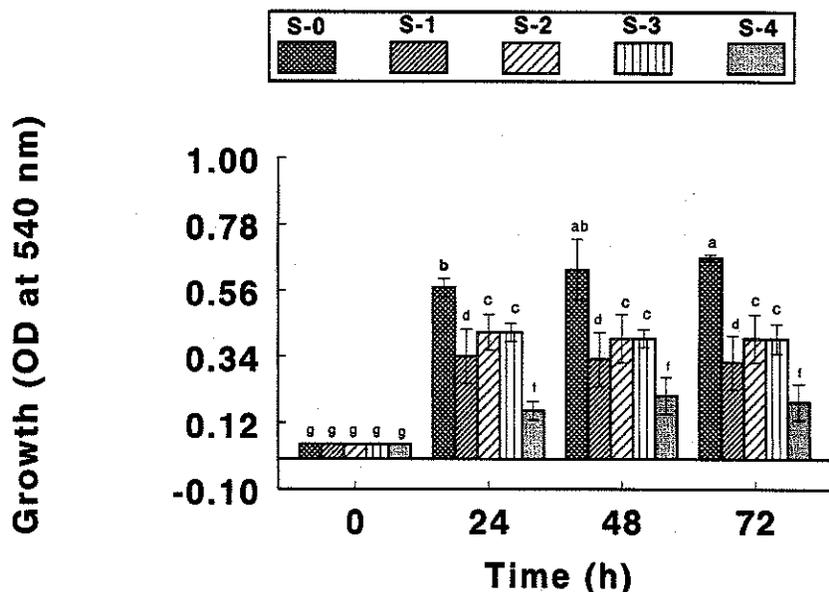


Fig. 2. Inhibitory effect of SLEB on the growth of *C. perfringens* spores grown in FTM at 37°C (pH 6.8), when the proportion of BHA in the SLEB treatment combinations was varied. FTM was inoculated with 10^4 – 10^5 spores per ml at time 0. SLEB combination treatments were S-0 (SLEB-75 ppm; 1:1:0.33), S-3 (SLEB-75 ppm; 1:1:0.66) and S-4 (SLEB-75 ppm; 1:1:1). Bar heights represent the mean of three independent experiments with triplicate subsamples. Means with the same letter superscript are not significantly different ($P < 0.05$). Error bars indicate the standard deviation, $n = 6$.

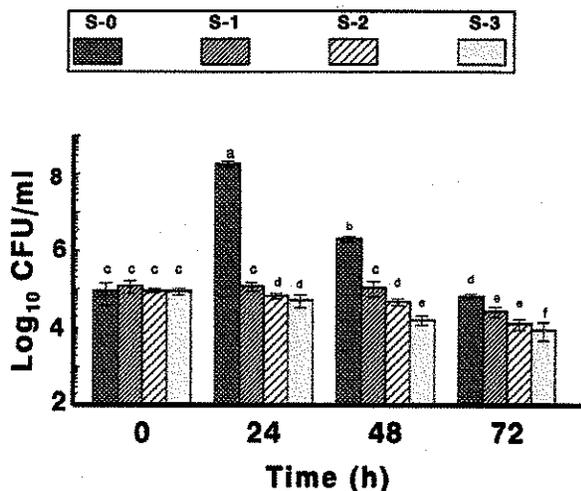


Fig. 3. Inhibitory effect of SLEB on the growth of *C. perfringens* spores grown in FTM (pH 6.8) and enumerated on TSC agar at 37°C, when the proportion of BHA in the SLEB combination was varied. FTM was inoculated with 10^3 – 10^5 spores per ml at time 0. SLEB combination treatments (SLEB-1:1:1 v/v/v) were: S-0 (SLEB 0), S-1 (SLEB-150 ppm, 1:1:1), S-2 (SLEB-150 ppm, 1:1:0.5) and S-3 (SLEB-150 ppm, 1:1:0.25). Bar heights represent the mean of three independent experiments with duplicate subsamples. Means with the same letter superscript are not significantly different ($P < 0.05$). Error bars indicate the standard deviation, $n = 6$.

treatments, the average O.D. (A_{540}) values for S-1 and S-4 were significantly different from each other and S-2 and S-3 ($P > 0.05$), but S-2 and S-3 did not differ significantly from each other ($P > 0.05$; Fig. 2). For some inexplicable reason, the mean A_{540} values for S-2 and S-3 were significantly larger than S-1 ($P < 0.05$; Fig. 2). This observation was unexpected, because treatments S-2 and S-3 contained higher concentrations of B; treatment S-1 contained only SL + E at 75 ppm.

At 150 ppm of SLEB, *C. perfringens* was cultured in FTM containing different SLEB combinations and plated on TSC agar for enumeration (Fig. 3). SLEB treatment combinations were S-0 (control), S-1 (1:1:1, v/v/v), S-2 (1:1:0.50, v/v/v) and S-3 (1:1:0.25, v/v/v). The mean \log_{10} number for the control (S-0) reached a maximum of $\log 8.25$ after 24-h incubation (37°C) and, subsequently, decreased to $\log 4.65$ after 72-h incubation (37°C; Fig. 3). However, during the same initial 24-h incubation period (37°C), the treatments mean \log_{10} values either remained unchanged or decreased from the

initial inoculum load of $\sim \log_{10} 5.0$ (Fig. 3). Thus, during 72-h incubation at (37°C) 150 ppm SL and E and different concentrations of B (0, 37.5, 75 and 150 ppm, respectively), no growth occurred among any of the treated samples, S-1, S-2 and S-3, albeit, the B concentration of SLEB in S-2 and S-3 was less than S-1, by 50% and 25%, respectively (Fig. 3). During the 72 h incubation period (37°C), the viability of *C. perfringens* in the treated samples never increased beyond the viability of 0 time spores (Fig. 3).

The results presented in Fig. 4 describe the efficacy of SLEB against the vegetative growth of *C. perfringens* in FTM. At 100 ppm SLEB (1:1:1, v/v/v), FTM broth cultures were inoculated with $\sim 8 \times 10^3$ spores per ml. Treatments included the negative control [C(-), bacteria only], positive control [C(+), bacteria + 100 ppm SLEB], T-1 [bacteria + 100 ppm SLEB added after 4-h incubation (37°C)] and T-2 [bacteria + 100 ppm SLEB added after 8-h incubation (37°C)]. The results

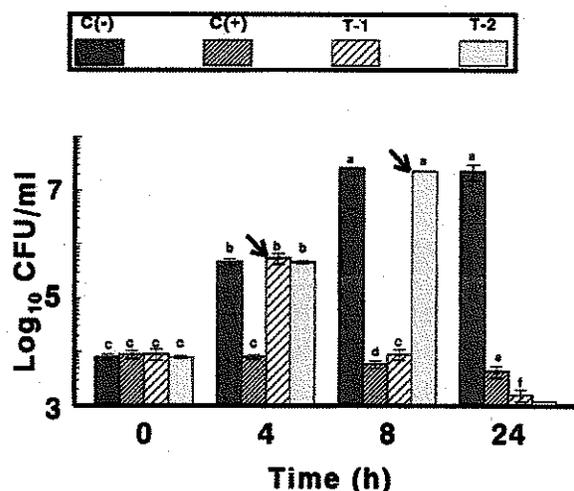


Fig. 4. Inhibitory effect of SLEB (100 ppm; 1:1:1, v/v/v) on the vegetative growth of *C. perfringens* in fluid thioglycollate medium (37°C, pH 6.8), when added at 4 and 8 h (arrows) after the initiation of growth. FTM was inoculated with 10^3 – 10^4 spores per ml at time 0. Cell enumerations were determined on TSC agar. SLEB combination treatments were: negative control [C(-), SLEB: 0 ppm added to FTM at 37°C] positive control [C(+), SLEB: 100 ppm added to FTM at 0 time]; Test-1 [T-1, SLEB: 100 ppm added to FTM after 4-h incubation, 37°C]. Bar heights represent the mean of two independent experiments with duplicate subsamples. Means with the same letter superscript are not significantly different ($P < 0.05$). Error bars indicate the standard deviation, $n = 4$.

showed that in the presence of 100 ppm of SLEB (1:1:1, v/v/v) *C. perfringens* growth was inhibited during 24-h incubation at 37°C [C(+), Fig. 4]. After 4-h incubation at 37°C, growth in T-1 had increased to \log_{10} 5.73, but after the addition of 100 ppm SLEB (1:1:1, v/v/v), the viable cell number decreased 1.8 logs to \log 3.95 at the end of an 8-h incubation (37°C) period (Fig. 4). After 24-h incubation, the cell viability had decreased to \log 3.15. Similarly, the \log viable count of T-2 had increased to \log 7.36 after 8-h incubation at 37°C. After the addition of 100 ppm SLEB (1:1:1, v/v/v) to FTM culture, the viable *C. perfringens* cell count decreased >4 log cycles to approximately 10^3 cells per ml during the next 16 h of incubation (37°C, Fig. 4). During the period that T-2 decreased more than four logs, the negative control [C(-)] remained essentially constant between 8 and 24 h ($P > 0.05$).

4. Discussion

The data presented in this study demonstrated how effective SLEB (1:1:1, v/v/v), as a combination preservative, worked against *C. perfringens* in laboratory media. Sikes (1995) showed the antimicrobial effectiveness of SLEB against *B. stearothermophilus* and *B. coagulans* in a food product. Current findings indicated that SLEB performs best when all of the components of the combination are present; however, Sikes and Whitfield (1992); Sikes and Flaig (1996) showed that two of the components of SLEB, SL and B, are effective antimicrobial agents against several foodborne bacteria when used alone, e.g., *L. monocytogenes*, *S. typhimurium*, *S. aureus* and *B. stearothermophilus*. But, at levels permissible in food, E (up to 500 ppm) has been shown to have no biocidal capability per se but functions as a potentiator in the SLEB combination system (Kagara, 1981).

On solid TSC agar, SLEB (1:1:1, v/v/v), at a concentration of ≥ 150 ppm (MIC), inhibited the germination and growth of a high concentration ($\sim 10^6$ spores per ml) of *C. perfringens* spores incubated (anaerobically) for up to 72 h at 37°C. However, in fluid thioglycollate medium (FTM), *C. perfringens* spores appeared to be slightly more sensitive to SLEB. For example, ~ 100 ppm of SLEB (1:1:1, v/v/v) effectively inhibited spore

growth (Fig. 1). No definitive explanation can be offered for this apparent difference.

Relative to the other spore-forming bacteria that have been evaluated for SLEB (1:1:1, v/v/v) sensitivity on solid laboratory media (Sikes, 1995), *B. stearothermophilus* and *B. coagulans*, *C. perfringens* was not as sensitive as *B. stearothermophilus* (MIC: 50 ppm) or *B. coagulans* (MIC: 85 ppm) but was more sensitive to SLEB than the nonspore-forming bacteria, *L. monocytogenes* (MIC: 200 ppm), *S. aureus* (MIC: 200 ppm) and *S. typhimurium* (MIC: 350 ppm). Based on these limited findings, it would appear that nonspore-forming bacteria (vegetative cells) are less sensitive to SLEB than either mesophilic or thermophilic spore-formers. However, when grown in FTM, it was demonstrated in the present study that \log vegetative phase cells of *C. perfringens* were as sensitive to SLEB (100 ppm; 1:1:1, v/v/v) as spores of *C. perfringens* (Fig. 4). The results also suggest that both gram-negative and gram-positive bacteria are sensitive to SLEB (Sikes and Whitfield, 1992); however, gram negative bacteria would appear to be the least sensitive.

The sensitivity of *C. perfringens* spores to SLEB was also determined, relative to the proportion of B in the treatment combinations. The results indicated that of the treatments combinations evaluated the most effective ratio was 1:1:1 (v/v/v), even at a concentration of 75 ppm (Fig. 2). Clearly, at 75 ppm, spore growth was initiated in the treated samples, reached a maximum O.D. (A_{540}) after 24 h incubation (37°C) and remained constant over the next 48 h. Conversely, the control samples reached maximum O.D. (A_{540}) after 48 h and remained constant over the next 24 h (Fig. 2). Other ratios of SLEB, where SL and E were at 75 ppm and B was at 25 (0.33) and 50 (0.66) ppm, were also capable of restricting *C. perfringens* growth but less effectively than in the combination in which all of the components were at 75 ppm (Fig. 2). Since B is the most restrictive additive of the combination, the results demonstrated that by increasing the proportion of SL and/or E to compensate for reduced usage of B may prove to be advantageous in certain situations.

At 150 ppm of SLEB, there was no apparent difference between treatment ratios, e.g., 1:1:1, 1:1:1/4 and 1:1:1/2, with respect to the extent of inhibition ($P > 0.05$). Since it was established that the MIC for SLEB (1:1:1, v/v/v) was 150 ppm on

TSC agar, the above observation was expected. The implications of the findings in this study are that SLEB, as a combination preservative, has flexibility. It was previously demonstrated that the proportion of individual components in SLEB can be adjusted to meet specific preservation requirements without compromising its integrity (Sikes and Whitfield, 1992). Thus, SLEB can be tailored to fit specific microbiological requirements of complex food systems. Additionally, SLEB would have application in hurdle technology.

Future SLEB research should include inoculated pack studies, in which more food products are tested. Studies should also focus on determining if the components of SLEB act synergistically in food systems and to what extent.

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