Growth of Spoilage Bacteria in Broth and Vacuum-Packed Bologna-Type Sausage at Fluctuating Temperatures and Low Temperature Storage

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ABSTRACT

The influence of fluctuating temperatures on some important spoilage bacteria, i.e., Brochothrix thermosphacta, Serratia liquefaciens and a Lactobacillus sp., as well as Vibrio sp. and Yersinia enterocolitica was determined in broth culture and in vacuum-packed Bologna-type sausage. Temperatures used were -2.0, 2.0 and 5.0°C. The influence of fluctuating temperatures varied considerably among the organisms studied. B. thermosphacta grew irrespective of temperature, numbers of S. liquefaciens decreased each time the product was stored at -2°C but grew at temperatures above 0°C. The effect on the Lactobacillus sp. depended on the initial storage temperature, whereas no significant difference in numbers of Y. enterocolitica were observed during fluctuating temperature storage. Generally the time-temperature combination was important and was the determining factor in the number of bacteria present after equivalent periods at the incubation temperatures above and below 0°C. The study indicated that with short periods of 2 to 3 d at each temperature, B. thermosphacta was favored. For long periods of 5 or 7 d at each temperature, lactics, S. liquefaciens and Vibrio sp. also increased to high numbers. B. thermosphacta, but neither S. liquefaciens nor Y. enterocolitica grew at a constant temperature of -2°C.

Few studies have been done on the time-temperature-tolerance (TTT) of foods at refrigeration temperatures and at temperatures just below 0°C, i.e., at superchilled temperatures where the water in the product is not frozen. If bacterial growth at superchilled temperatures is inhibited, storage of vacuum-packed meat products at these temperatures and at fluctuating temperatures involving subzero temperatures could prove beneficial. A study by Bøgh-Sørensen and Zeuthen (3) considered storage of vacuum-packed Bologna, smoked and cooked pork loin, and unsmoked pork filet at -3.5, 2 and 4°C or at alternating temperatures of -3.5 and 4°C. Only total counts, Brochothrix thermosphacta and lactics were examined, and no effect on B. thermosphacta was observed because of low counts during the entire storage period (3). Similarly, there was no effect of fluctuating temperature on the lactics. Neither lactics nor B. thermosphacta grew at a constant temperature of -3.5°C. Total counts increased during storage at fluctuating temperatures in the two products. Sometimes, but not always, numbers increased at 4°C and decreased at -3.5°C. Hence, the effect of fluctuating temperatures or superchilled temperature could not be determined. The numbers of lactics did not increase during storage of vacuum- or nitrogen-packed frankfurters at -4°C (21) nor was any significant increase in lactics observed at -4°C in vacuum- or nitrogen-packed meat loaves (12). The number of lactics on veal chucks increased ca. 1 log10 unit during 2 wk at -4°C, with no further development during the following 8 wk (11). Growth of lactic acid bacteria was observed at 0°C.

The purpose of this study was to investigate the effect of fluctuating temperatures on B. thermosphacta, Serratia liquefaciens and a lactic acid bacterium in a vacuum-packed cooked, cured meat product and in broth cultures. The TTT concept states that the effects of time and temperature are cumulative considering the whole storage period, i.e., the sequences of storage temperatures are without influence on the product. Two other bacteria, an atypical Vibrio sp. and Yersinia enterocolitica, also were included in the study. Previous studies have not been consistent with respect to specific microorganisms, hence, it was decided to use pure cultures of known bacteria as inocula for this study.

MATERIALS AND METHODS

Microorganisms

Brochothrix thermosphacta (strain No. 153), S. liquefaciens (strain No. 155) and Lactobacillus sp. (strain No. 4, an atypical Streptobacterium) and a non-motile, penicillin-sensitive (atypical) Vibrio sp. (5) were obtained from our laboratory collection. Yersinia enterocolitica (serotype 0:3) was obtained from Dr. S. G. Christensen (Royal Veterinary and Agricultural University, Denmark). Bacteria were grown in brain heart infusion broth (BHI, Difco) at 20°C and serially diluted in sterile peptone-saline water (0.1% Bacto-peptone with 0.85% NaCl). The stor-
age experiments were done in BHI with 4% NaCl added, or Bologna-type sausage from a commercial manufacturer. Broth studies were done with individual cultures in 100-ml screw cap flasks containing 20 ml of medium. Flasks were inoculated with 0.1 ml of a fresh culture (logarithmic growth phase) of the appropriate bacterium and incubated under stationary conditions. Experiments with sausages were done by removing the casings in the laboratory using aseptic procedures, and slicing sausages with a slicer which was previously disinfected. Five slices (50 g) were placed in polyethylene-polyamid bags (Otto Nielsen Ltd, Denmark). After incubation with 0.5 ml of an appropriate dilution, the bags were vacuum packed.

**Storage**

Storage temperatures were -2.0, 2.0 and 5.0 ± 0.1°C. Fluctuations in temperature were accomplished by moving flasks and vacuum packages between appropriate incubators. Time-temperature combinations differed for some of the experiments because of practical problems. An example of the notation used is 3-2-2-3 d at -2°C/5°C, which means the culture was incubated for 3 d at -2°C, then for 2 d at 5°C, then 2 d at -2°C, and finally 3 d at 5°C. The notation 5°C/-2°C implies initial storage at 5°C followed by storage at -2°C. *B. thermosphacta*, *S. liquefaciens* and *Y. enterocolitica* were also studied at constant temperatures.

**Bacterial analyses**

Serial dilutions of broth cultures of *B. thermosphacta* and *S. liquefaciens* were plated onto plate count agar (PCA, Difco). Analyses of vacuum-packed samples were made by homogenizing the whole sample with 150 ml of sterile NaCl-peptone water in a Stomacher, and plating serial dilutions onto PCA. The *Lactobacillus* sp. was plated onto all purpose medium with Tween (APT, Difco). Experiments with *Vibrio* sp. were done using similar procedures, except that the sodium chloride concentration was adjusted to 4% in NaCl-peptone medium and in PCA. After pasteurization, vegetative bacteria were not detected in sausages. The few bacilli spores present (ca. 3/g) did not grow at the temperatures used. Hence, there was only growth of the inoculated bacterial culture in the packages, and only one plating medium was necessary.

**Sodium chloride and moisture determinations**

The concentration of sodium chloride was determined by Volhard titrations (2) and moisture content was determined by drying at 104°C (1).

**Statistical analyses**

When broth cultures or vacuum packages with different initial storage temperatures were exposed to equal time-temperature combinations, microbial counts were subjected to an analysis of variance (22).

**RESULTS AND DISCUSSION**

Results of growth experiments are shown in Figures 1 through 7. Sodium chloride and moisture content in the Bologna-type sausage were 2.3 and 59.1%, respectively, which was equivalent to a salt concentration in the water phase of 3.9%. At this salt concentration, the product did not freeze during subzero storage.

An initial short period of 3 d at -2°C had no effect on the number of *B. thermosphacta*, whether in broth culture (Fig.1) or in vacuum-packed Bologna (Fig. 2). When shifting the temperature to 5°C for 2 d, the bacteria started growing. Once growth started, *B. thermosphacta* continued growing when the temperature was reduced to -2.0°C again. Hence, a TTT (time-temperature-tolerance) combination of 3-2-2-3 (Fig. 1) or 3-4-4-3 (Fig. 2) d shifting between -2.0 and 5.0°C resulted in the same numbers after 10 d whether storage was started at -2.0 or 5°C (P≤0.05). *B. thermosphacta* also grew, but more slowly, at a constant temperature of -2.0°C (Figs. 1 and 2). Therefore, when using the combination of 7-7 at -2°C/2°C, i.e., 7 d at -2°C followed by 7 d at 5°C, enough time was available at -2.0°C to initiate growth, and the numbers were only slightly lower after 14 d when the...
initial storage temperature was at -2°C than at 5°C (Fig. 2) (P<0.05). Studies at constant temperature of -2, 2 and 5°C revealed that although *B. thermosphacta* grows at all temperatures, storage temperature had an important effect on the rate of bacterial development. Hence, the time to reach $10^7$ *B. thermosphacta/g* at -2, 2 and 5°C was about 4, 8 and 15 d, respectively (Fig. 1). Results in Bologna-type product at -2 and 2°C were similar (Fig. 2).

*Serratia liquefaciens* grew each time the broth culture or packages of Bologna were incubated at 5°C (Figs. 3 and 4), whereas the numbers decreased each time the samples were stored at -2.0°C, when using the TTT combination of 3-2-2-3 d or 3-4-4-3-4-4-3 d. *S. liquefaciens* was also studied in Bologna sausage using periods of 7 d at each temperature (-2°C/2°C and -2°C/5°C or vice versa, Fig. 5). These figures indicate that when the numbers of *S. liquefaciens* were high ($10^7$ to $10^9$/g) and near the stationary phase, the bacteria were less susceptible to the lethal effect of shifting to -2°C. Hence, once in the early logarithmic phase of growth, a shift to subzero temperature results in a greater decline in numbers than when the bacteria are in the late logarithmic phase or stationary phase of growth. This means that the TTT concept is not valid for *S. liquefaciens* during logarithmic growth because there is not a cumulative effect of time and temperature. After a total of 10 d (5 d at each of two temperatures, i.e. -2 and 5°C) the bacterial numbers in the series starting at -2°C and those starting at 5°C were not similar (Fig. 3). This also occurred for *S. liquefaciens* in vacuum-packed Bologna after 14 d (3+4 d at -2°C and 3+4 d at 5°C) of storage. The two curves do not meet until the stationary phase is reached (after 25 to 28 d). With periods of 7 d at each temperature in Bologna sausage, there was enough time for growth at 5°C (but not at 2°C) to compensate for the period at -2°C, resulting in equivalent numbers of bacteria in the two series after 14 d and 28 d (Fig. 5). *S. liquefaciens* did not grow during 3 to 4 wk of storage at a constant temperature of -2°C (Figs. 3 and 4). Comparing the data for growth at 5 and 2°C (Fig. 3), $10^7$ bacteria/g of Bologna was reached after 7 and 14 d, respectively. When the storage temperature was -2°C, the number of bacteria was constant.
Growth of Vibrio sp. in broth was monitored during 3-4-4-3 d at -2 and 5°C and also at -2 and 2°C (Fig. 6). For both combinations, the number of Vibrio sp. decreased during storage at -2°C, whereas bacteria grew when the temperature was shifted to 2 or 5°C. However, bacterial numbers again decreased during storage again at -2°C. In contrast, initial storage at 2 or 5°C resulted in growth which continued, although at reduced rate, when cultures were shifted to -2°C. Maximum numbers were reached after the 3+4 d combination at -2°C/5°C, but this did not occur for the -2°C/2°C combination. Initial storage at -2°C resulted in lower maximum bacterial numbers after 3-4-4-3 d than did initial storage at 5°C.

Experiments with Lactobacillus sp. for 7-7-7-7 d at -2°C/5°C or 5°C/-2°C revealed that initial storage at -2°C resulted in a decline in bacterial numbers, as did the next period of storage at subzero temperature, whereas initial storage at 5°C resulted in increased numbers of Lactobacillus sp. during periods at -2°C. However, after 14 and 28 d (equal time at the two temperatures), the number of lactobacilli was not significantly different (P>0.05) in the two series. The numbers of Y. enterocolitica during 28 d of storage were relatively constant whether storage began at -2 or 5°C or if the temperature was constant at -2°C (Fig. 7).

The TTT concept was proposed for refrigerated and superchilled temperatures, i.e., that there is a cumulative effect of time and temperature on bacterial growth irrespective of initial temperature. The purpose of this study was to determine, using single cultures, if this concept was valid relative to logarithmic growth and maximum numbers of bacteria after equivalent storage periods at temperatures of -2.0°C and 5°C. Furthermore, the influence of storage at constant temperatures of -2, 2 and 5°C was studied for B. thermosphacta, S. liquefaciens and Y. enterocolitica (-2°C only).

Results obtained at subzero temperature were interesting. Although growth of both gram-positive and gram-negative bacteria below 0°C has been reported (8), this study revealed that only B. thermosphacta and not the two gram-negative bacteria, grew below 0°C. B. thermosphacta and S. liquefaciens are important spoilage bacteria (15,16) and the present results indicate that storage of food a few degrees below zero would favor growth of B. thermosphacta. Abundant growth was also reported at -1°C on packaged lamb chops (14); however, -2°C is the lowest reported growth temperature for B. thermosphacta. Whether B. thermosphacta would also grow at -2°C in a frozen product can not be determined from these results. Growth at superchilled temperature, but not at the same temperature in the frozen state, was reported for Bacillus sp. (10). S. liquefaciens was inhibited at -2°C, and similar results were obtained with other gram-negative bacteria at -4°C (21). Although growth of Y. enterocolitica is inhibited by a competing flora at refrigeration temperature (18), this bacterium grows rapidly at 2 and 5°C when present in pure culture (17). Slow growth of Y. enterocolitica has been reported at 0°C (7). The present study revealed no growth at -2°C, indicating that growth would also be inhibited on frozen product at ≤-2°C because of the lower water activity when the water is in the frozen state (4).

Initial storage temperature was important and influenced the number of bacteria obtained after the next temperature shift. This effect was especially marked with short storage periods at each temperature. At longer storage periods (7 d), there was sufficient time to recover and grow during the next period. A temperature shift to subzero temperature was more important when the bacteria were in the logarithmic phase of growth, where bacterial numbers often decreased, whereas at a constant
temperature of -2°C, numbers (S. liquefaciens and Y. enterocolitica) remained constant even though these bacteria were in the logarithmic phase of growth at time of inoculation. In experiments with bacteria growing in the exponential phase, rapidly cooling the culture caused a decrease in viable numbers which was related to "cold shock" (20). Greatest loss of viability occurred when the culture was chilled very rapidly, and not at a rate of ca. 1°C/min (13). The actual temperature difference was also important, i.e., the greater the temperature differential the greater the loss of viability. The study revealed that a temperature shift to -2°C had a greater effect on gram-negative bacteria than on gram-positive bacteria.

The reason for including Vibrio sp. in the study was that this bacterium is more resistant to NaCl than S. liquefaciens (16). The Vibrio sp. was an atypical Vibrio which may be found in vacuum-packed cooked, cured meat products, and can grow to quite high levels (15). The organism may contribute to spoilage of these products (5,6). The Vibrio sp., however, did not differ markedly from S. liquefaciens.

Growth of all of the important spoilage bacteria, i.e., B. thermosphacta, S. liquefaciens and Lactobacillus sp., occurred during storage at fluctuating temperature. However, the study revealed that after a total storage period of 4 wk (partly above and below 0°C) the bacterial numbers essentially will be the same regardless of an initial temperature of -2 or 5°C. Bacterial numbers will be at a higher level for a longer period if the initial storage temperature was 2 or 5°C than at -2°C. Because spoilage requires the development of large numbers of bacteria over a period of time, the information obtained by this study is valuable and should be further investigated using both microbiological and organoleptic analyses.

REFERENCES