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Effect of redox potential on *Bacillus subtilis* and *Bacillus licheniformis* in broth and in pasteurized sausage mixtures

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Bacillus licheniformis (a facultatively anaerobe) and *B. subtilis* (aerobic) may cause spoilage in certain meat products pasteurized in hermetically sealed containers if these are stored with inadequate refrigeration. After having optimized a method for determination of the redox potential in sausage mixtures, we investigated the contribution of low redox potential (E_h) and anaerobiosis to the inhibition of these bacilli. With the E_h values encountered in pasteurized meat products there was no effect of the redox potential on growth of *B. licheniformis*, either in model broths or in mildly heated bologna-type sausage mixtures. When cultured in a model broth with head space, *B. subtilis* grew to about 10^7/ml and was little affected by the initial E_h value (range between +250 and −150 mV) and the initial dissolved oxygen concentration. During exponential growth of this bacterium in the absence of oxygen, the E_h value increased by about 180 mV. In sausage mixture with air inclusions, *B. subtilis* attained slightly higher cell densities than in mixtures protected against air uptake but it was subsequently overgrown by facultative anaerobic bacilli. We conclude that knowledge of the E_h value of a meat product does not permit a prediction of the growth potential of bacilli in pasteurized meat products, and that a sufficient heat treatment and proper refrigeration are essential to control these bacteria.

Key words: Redox potential; *Bacillus* spp.; Growth; Sausage

Introduction

To predict the shelf life of meat products, it is essential to know the parameters that influence the survival and growth of microorganisms in the product. Important intrinsic factors are the pH and a_w values both of which can be determined by reliable methods (Rödel et al., 1988). There is evidence that the growth of microorganism in food is also influenced by the redox potential (Barnes and Ingram, 1955; Leistner and Wirth, 1965; Wirth and Leistner, 1969; Hofmann, 1974;

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Wojciechowski et al., 1976; Wilberg and Nilsson, 1980). However, because of problems in obtaining and interpreting reliable data, this factor has not been given major attention.

The shelf life of meat products that are pasteurized in hermetically sealed containers and stored at abuse temperatures is often limited by the growth of *Bacillus* and *Clostridium* species whose spores survived the heat treatment. Spices are major source of *Bacillus* spores (Neumayr, 1983), and the average spore load of sausage mixtures prepared with untreated spices has been estimated to be about 5000 per gram (Hechelmann and Leistner, 1987). Berkel and Hadlok (1976) found that *Bacillus subtilis, B. pumilus* and *B. licheniformis* predominate in pasteurized meat products. In the absence of oxygen, there is little if any growth of the former two species while *B. licheniformis* and *B. cereus*, when cultivated in suitable culture media, attain cell densities of about 10^8 per ml even under anaerobic conditions (Neumayr, 1983). However, when pasteurized canned meat products containing *Bacillus* spores were incubated without refrigeration, the *Bacillus* count frequently remained below 10^6 per gram and the products showed only minor signs of spoilage (Leistner et al., 1980; Hechelmann and Leistner, 1984; Lücke, 1984). Furthermore, Bell and De Lacy (1982) observed that *B. licheniformis* grew only on the surface of luncheon meat pasteurized in oxygen-permeable film or in inclusions of air and could be inhibited by storing the packs under hydrogen. These data indicate that the growth of bacilli in meat products is influenced by the partial pressure of oxygen and possibly other electron donors and acceptors present in meat.

In contrast to the redox potential, the oxygen partial pressure cannot be readily measured in solid substrates with standard methods. Hence, we investigated first of all the influence of the redox potential (E_h) on the growth of *Bacillus* species in culture media and in bologna-type sausage.

Materials and Methods

Bacterial strains

The *B. licheniformis* strains B 29, B 49, B 78, B 123 and B 126 and the *B. subtilis* strains, B 1, B 8, B 9, B 12, B 13, B 45, B 54, B 55, B 56 and B 74 from the culture collection of the Federal Centre for Meat Research, Kulmbach were used. Spores of these strains were generated on nutrient agar (Merck) to which 10 mg $MnSO_4 \cdot 2H_2O$ had been added per liter. After up to 3 days incubation at 37 °C, spores were rinsed off the surface, washed twice with distilled water and stored frozen. For the experiments, spores of each species were pooled and the resulting suspension adjusted to give an initial spore count of approx. 10^4 per ml. Before inoculation of culture media, spores were heat activated for 10 min at 70 °C.

Culture media and growth conditions

Modifications of the medium "RM" of Neumayr (1983) were used. These contained 2% (w/w) peptone from meat, 1% (w/w) meat extract powder, 15 mM glucose, 50 mM DL-lactic acid, 25 mM sodium phosphate and variable amounts of

sodium chloride (2–6% (w/w) to give a_w values between 0.981 and 0.958) and of sodium nitrite. The pH was adjusted to 6.2 unless indicated otherwise. The medium was then equilibrated with air or oxygen-free gas as defined below. Subsequently, the initial redox potential was adjusted by adding variable amounts of a freshly prepared solution of cysteine hydrochloride to give final concentrations of 0.05–0.2% (w/w).

Growth experiments with *B. subtilis* ('syringe cultures') were carried out in sterile polypropylene syringes (nominal capacity, 30 ml) completely filled with RM medium containing 3% (w/w) NaCl. To remove dissolved oxygen, an anaerobic cabinet (Forma Scientific, Marietta, U.S.A.) containing nitrogen, hydrogen and CO_2 in the ratios of 85 : 10 : 5 (v : v : v) was used to equilibrate the medium, to supplement it with cystein, inoculate it and fill it into the syringes which were then sealed by punching the needle into a rubber stopper. This resulted in an initial oxygen content of less than 2% of air saturation. To avoid the interference of air with the measurement of E_h and dissolved oxygen, the piston was removed in an argon atmosphere. Incubation temperature was 37 °C. In sterile controls the oxygen content did not increase significantly throughout the experiment (in 8 days from 4.2×10^{-3} to 0.11 ml O_2/30 ml medium).

B. licheniformis was grown in RM medium with various additions as shown in Table I in a fermenter (working volume, 850 ml; BCC, Göttingen, FRG) at 27 ± 1 °C under a slow flow of oxygen-free nitrogen. Viable counts of bacilli were determined on Standard I nutrient agar (see below).

Bologna-type sausage mixture was prepared from 25% lean beef, 25% lean pork, 28% pork back fat, 15% ice, 5% skim milk powder and 2% soy protein (all % w/w). Per kg of this mixture, 16 g nitrite curing salt (to give an initial nitrite concentration of approx. 75 mg $NaNO_2$/kg), 4 g NaCl, 0.5 g sodium ascorbate and 3 g sodium diphosphate were added. The mixture was comminuted until it no longer contained visible meat and fat particles. It had an a_w of 0.970–0.974 which proved adequate to suppress the growth of clostridia during the incubation period of 4 weeks. In each experiment, batches 1 and 2 were prepared and inoculated in a vacuum cutter (Stephan, Hameln, F.R.G.) whereas a standard cutter (Müller, Saarbrücken, F.R.G.) was used to prepare and inoculate batch 3. Before filling into cans (diameter 75 mm; capacity 200 g), batch 2 was re-aerated by several additional turns in the opened cutter. The cans were heated at 95 °C in an open water bath to give F_0 values in the order of 0.02 and subsequently stored at 25 °C.

Analytical methods

E_h values were determined using combined redox electrodes with platinum diaphragm, calomel as lead-off element and 3.5 M KCl as electrolyte (type Pt61; Schott-Geräte, Hofheim, FRG), joined to a millivolt meter (type 537; WTW Weilheim, FRG). All millivolt values were transferred to E_h'-values (at pH 7.0) using the equation $E_h' = E_h + E_{h_{cal}} + E_N(px - 7.0)$ (Leistner and Mirna, 1959). E_h is the measured potential (in mV), $E_{h_{cal}}$ is the potential (in mV) between the calomel electrode and the standard hydrogen electrode at the temperature of measurement, E_N is the Nernst factor (in mV) at this temperature, and px is the pH value during

measurement. The pH was measured by means of a combined electrode equipped with three platinum diaphragms (Schott no. N58), and % air saturation in stirred liquid media was analysed using a Triox oxygen electrode (WTW, Weilheim, F.R.G.) coupled to a WTW OXI 2000 device. During growth of *B. licheniformis* in the fermenter, the E_h value of the medium was monitored continuously. Prior to the insertion into the fermenter, all electrodes were sterilized by immersion into 6% sodium hypochlorite for 10–30 min, followed by rinsing with 1% sodium thiosulfate and with water. Prior to E_h measurement in 'syringe cultures' and in sausage mixtures, the platinum surface of the electrode was cleaned with finely grained polishing paste (WTW, Weilheim, F.R.G.) and the electrode calibrated using the redox buffer no. 209881 (Ingold, Steinbach, F.R.G.). In sausage mixtures, up to 2 h were required to obtain stable readings.

An electrolytic device (Rotronic, Zürich, Switzerland) was used to determine water activity (Rödel et al., 1979). To obtain an estimate of the air content the specific gravity of the sausage mixtures was measured by the method of Mahling (1965). For microbiological analysis sausage samples of 20 g were homogenized in 180 ml diluent (0.85% NaCl, 0.1% peptone). Bacilli were counted on Standard I nutrient agar (Merck, Darmstadt, F.R.G.) after 2 days at 37 °C. The nitrite content of the culture media was assayed using test sticks (Merckoquant no. 10007; Merck, Darmstadt).

Results

Observations from E_h measurements in sausage mixtures

To obtain stable and reproducible values when measuring E_h in sausage mixtures, it proved necessary to polish the platinum surface prior to each measurement. Furthermore, the hole punched for the insertion of the electrode had to be wetted by a drop of 10 mM KCl to ensure good electrical contact between the product and the electrode. Without these precautions, 'phantom potentials' were frequently registered or the time to reach stable reading was extended considerably. In contrast, the presence of air in the headspace did not interfere with the measurement. The results from these studies, which are not included, confirm earlier observations (Jacob, 1970; Brown and Emberger, 1980).

In liquid media reproducibility of E_h measurements was very high with a coefficient of variation of 0.002 ($n = 10$). When samples of sausage mixture from the same batch were analysed, the coefficient of variation was 0.07 ($n = 7$).

Effect of redox potential on growth of B. subtilis in liquid media

B. subtilis is known as an obligate aerobe even though limited anaerobic growth may occur if certain electron acceptors are available (Claus and Berkely, 1986). In RM broth with reduced ($< 2\%$ of air saturation) content of oxygen, we observed an increase of the cell density from 2×10^4/ml to a final value of approximately 10^7/ml within the first day (Fig. 1). There was only a slight increase in turbidity, and growth would probably have been scored 'negative' if examined visually.

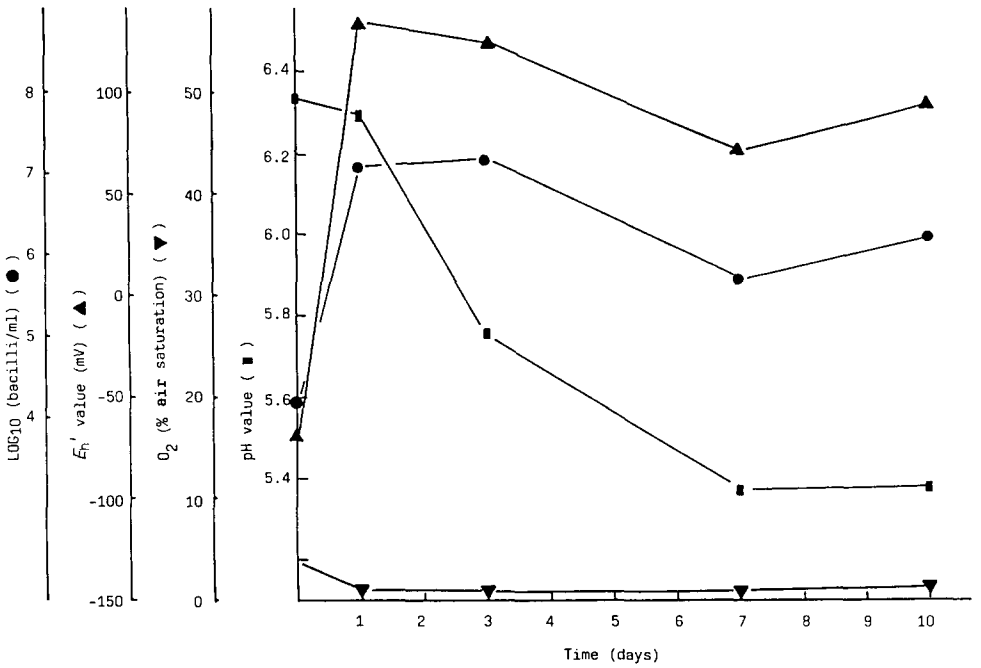

Fig. 1. Time course of redox potential (▲), pH (■), air saturation (%) (▼) and count of *B. subtilis* (●) in oxygen-free RM broth. The culture was grown in headspace-free syringes at 37 ° C.

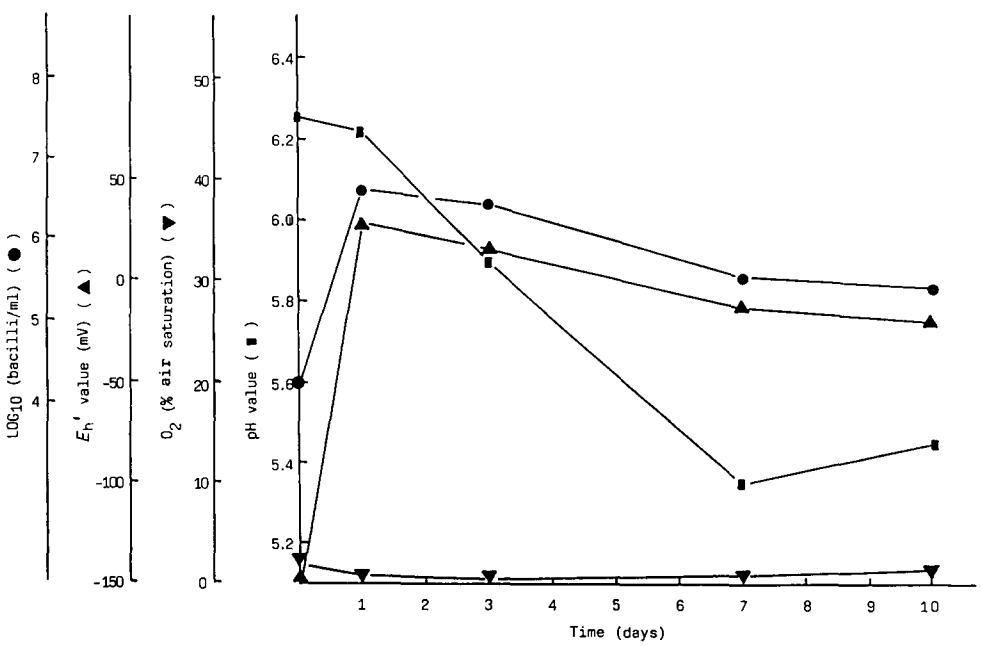

Fig. 2. Time course of redox potential (▲), pH (■), air saturation (%) (▼) and count of *B. subtilis* (●) in oxygen-free RM broth supplemented with 0.2% cysteine hydrochloride. The culture was grown in headspace-free syringes at 37 ° C.

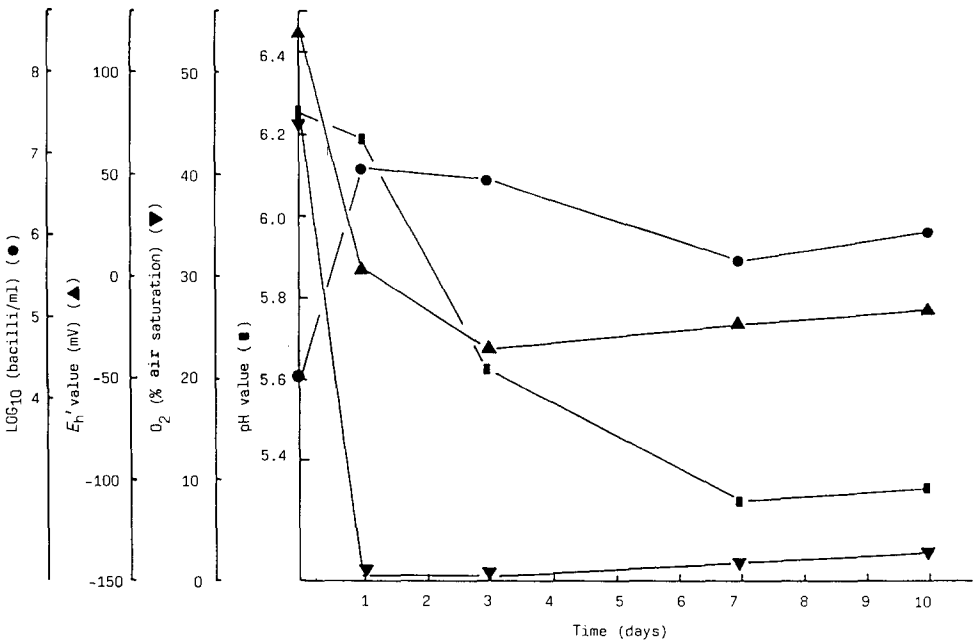

Fig. 3. Time course of redox potential (▲), pH (■), air saturation (%) (▼) and count of *B. subtilis* (●) in RM broth supplemented with 0.2% cysteine hydrochloride and initially containing dissolved oxygen (46% of air saturation). The culture was grown in headspace-free syringes at 37 °C.

Surprisingly, the E_h' value increased by about 180 mV. Addition of 0.2% cysteine hydrochloride lowered the E_h' value by about 90 mV throughout the experiment and gave a slightly lower cell yield (Fig. 2). When the medium contained higher levels of oxygen (> 50% of air saturation) at the initiation of the experiment (Fig. 3), oxygen was rapidly consumed and did not significantly improve the cell yield. The E_h' value decreased from +120 to −40 mV which is close to the final E_h' value attained after initial removal of oxygen. It appears from these experiments that within the range of +120 to −150 mV, the E_h value has little if any effect on the growth of *B. subtilis* in liquid media with no headspace.

Effect of redox potential on B. licheniformis in liquid media

B. licheniformis was grown at 27 °C in a 1-l fermenter in the absence of oxygen. As indicated in Table I, the medium (RM) was adjusted to various a_w and E_h' values by means of sodium chloride and cysteine, respectively, to levels similar to those found in bologna-type sausages. In experiments 6, 7 and 8, RM was further supplemented with filter-sterilized nitrite to give initial concentrations of 50 and 100 mg $NaNO_2$/l. The latter concentration resembles the initial nitrite concentration in the water phase of a commercially prepared bologna-type sausage. Within the range of E_h values between −30 and −210 mV, no effect of the redox potential on the growth of *B. licheniformis* was observed and the bacterium grew with doubling

TABLE I

Summary of growth experiments with *B. licheniformis* in RM broth (pH 6.2) with various additives under anaerobic conditions at 27 °C

Experiment no.	Additives			a_w	E_h' value max/min.	Growth within 14 days [a]
	NaCl (%)	Cysteine hydrochloride (%)	$NaNO_2$ (mg/kg)			
1	2	0.05	–	0.981	−30/−130	+
2	2	0.1	–	0.981	−109/−170	+
3	2	0.2	–	0.981	−194/−218	+
4	4	0.2	–	0.968	−150/−197	+
5	6	0.2	–	0.958	−201/−210	+
6	6	0.2	50	0.958	−185/−200	+
7	6	0.2	100	0.958	−99/−149	−
8	6	0.05	100	0.958	7/−100	−

[a] +, increase of cell density by three log cycles; −, no increase in cell density.

times of 7–8 h to densities of approximately 10^8/ml and lowered the pH to 5.7–5.8 at the a_w values tested (data not shown). However, as previously observed by Neumayr (1983), nitrite considerably delayed growth from spores of *B. licheniformis* under anaerobic conditions: after addition of 100 mg sodium nitrite/l, growth was inhibited for more than 2 weeks although the nitrite content of the medium decreased to below 10 mg $NaNO_2$/l within the first 4 days of incubation. From these experiments, it appears that nitrite addition contributes significantly to the inhibition of surviving *B. licheniformis* spores and so will the storage temperature, while growth is unaffected by the E_h and a_w values encountered in pasteurized meat products. However, the fate and antimicrobial activity of nitrite added to broth and meat may differ because of different forms and concentrations of nitrosable iron and sulfhydryl groups (Cassens et al., 1979).

Effect of redox potential on growth of bacilli in pasteurized sausage mixtures

The *Bacillus* spores used had a rather low heat resistance with D values of about 1–3 min at 100 °C (unpublished results). Hence, the F_0 values used (approx. 0.02) was expected to give a 10 to 100-fold reduction of the spore inoculum. To inhibit the development of clostridial spores that are much more resistant to heat, the water activity of the mixture was lowered to about 0.97. Although the air content of the mixtures (as estimated from the specific gravities of the mixtures) could be varied considerably using different comminution techniques, aeration of the mixtures had little effect on the redox potential which varied between +68 and −83 mV. The specific gravity varied between 0.95 and 1.06 g/cm^3. Growth of *B. licheniformis* at 25 °C occurred in all batches and at all E_h' values. Fig. 4 shows, as an example, the time course of the cell numbers of *B. licheniformis* and of the E_h' value during storage at 25 °C of canned pasteurized bologna-type sausage mixture prepared under vacuum and subsequently aerated.

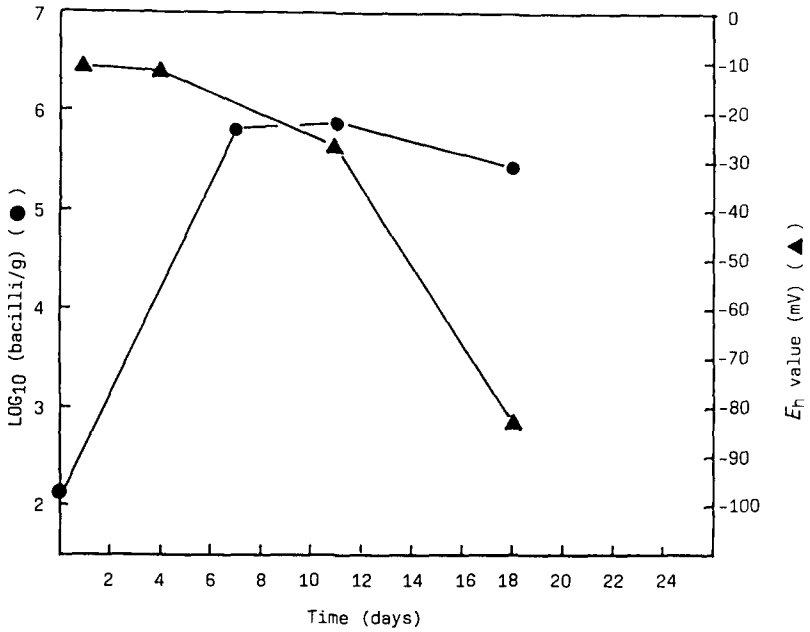

Fig. 4. Time course of redox potential (▲) and count of *B. licheniformis* (●) in canned bologna-type sausage mixture heated to $F_0 = 0.02$ and stored at 25 °C. The mixture was prepared under vacuum and subsequently 'aerated' by several additional turns in the opened cutter.

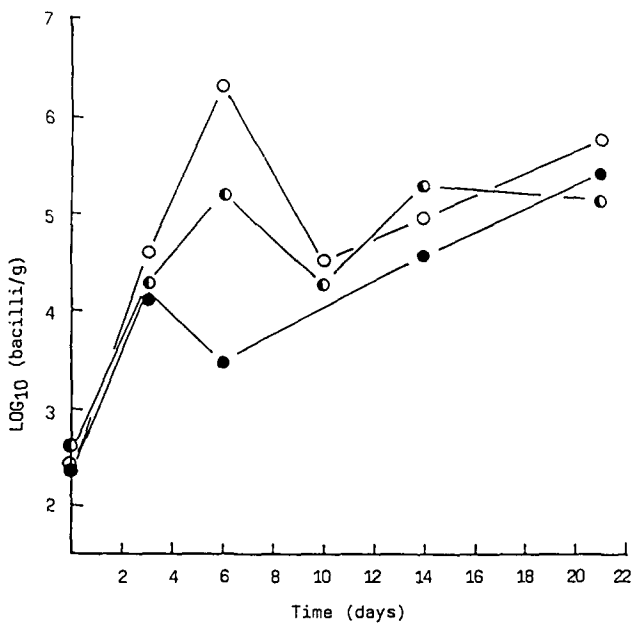

Fig. 5. Growth of bacilli in canned bologna-type sausage mixture heated to $F_0 \approx 0.02$. (●), processed under vacuum; (◐), processed under vacuum, subsequently 're-aerated'; (○), processed in the presence of oxygen. Until day 7, the *Bacillus* flora mainly consisted of the inoculum (*B. subtilis*); later, *B. licheniformis* and *B. cereus* became dominant. Incubation took place at 25 °C.

As shown in Fig. 5, there was some growth of *B. subtilis* in all batches of bologna-type sausage mixture within the first 3 days of storage at 25° C, irrespective of the presumptive initial air content of the mixture. After 6 days storage, however, the *Bacillus* counts appeared to be higher in the batches with the presumed higher content of air. Upon further storage, *B. subtilis* was overgrown by facultative anaerobic bacilli (*B. cereus, B. licheniformis*) present in the indigenous flora. This resulted in similar cell densities (approx. 10^5/g) in all batches after 2–3 weeks.

Discussion

We were able to improve the reproducibility of E_h measurements in sausages and shorten equilibration time to less than 2 h by employing a standardized polishing procedure and using small amounts of a diluted electrolyte to ensure optimal electrical contact between sample and electrode. The state of the electrode surface influences the rate by which substances in the sample feed electrons to or withdraw electrons from the electrode (Jacob, 1970; Brown and Emberger, 1980). In contrast to many reports in the literature, oxygen did not interfere with our E_h measurements in sausages. This may be due to our efforts to avoid introduction of air inclusions while inserting the electrode, to the slow diffusion of molecular oxygen into the sausage mixture and its dissipation by residual nitrite.

In the range representative for meat products (E_h' between 0 and −200 mV), we did not observe any marked effect of the initial redox potential on the growth of *Bacillus subtilis* and *B. licheniformis*. This was true both for liquid media (E_h' value adjusted with molecular oxygen and/or cysteine) and for bologna-type sausage mixtures prepared with different amounts of air inclusions. Consequently, the microbiological stability of pasteurized meat products cannot be predicted from measured redox potentials. Similarly, Montville and Conway (1982) failed to observe a major effect of the redox potential on the growth of *Clostridium botulinum* in various canned foods.

Most of the reports on the effect of redox potential on microorganisms deal with clostridia. There is evidence (summarized by Morris, 1976) that these bacteria not only require the absence of oxygen but also 'reducing conditions', i.e., low redox potentials. Barnes and Ingram (1955) demonstrated that *C. perfringens* only initiates growth in fresh meat after the E_h' value has dropped to below −36 mV, and it was shown that this clostridium (Mead, 1969) as well as *C. botulinum* (Lund and Wyatt, 1984) required low redox potentials for growth at elevated salt concentrations. However, compounds used to adjust the redox potential may have an effect on the growth of anaerobes as well, which cannot readily be distinguished from the effect of E_h value per se (Smith and Pierson, 1979). Furthermore, growing cultures may consume and form compounds that react with redox electrodes and thus change the redox potential. Most investigators observed a decrease of the redox potential during exponential growth of various bacteria under both anaerobic and aerobic conditions (Mead, 1969; Tabatabai and Walker, 1970; Oblinger and Kraft, 1973; Douglas et al., 1973). Hence, only the effect of the initial E_h value on initiation of

growth can be studied in batch culture; to keep the redox potential at a constant level, the organisms must be grown in continuous culture under carbon or oxygen limitation (Kjaergaard, 1976). As expected, we found that consumption of dissolved oxygen by *B. subtilis* markedly diminished the E_h' value (Fig. 3); however, exponential growth of *B. subtilis* in the absence of oxygen leads to an increase of the redox potential to approximately the same level as attained in the initially 'aerobic' cultures. (Figs. 1, 2). In the absence of precise knowledge which compounds are metabolized and formed by *B. subtilis* and *B. licheniformis* under anaerobic conditions and how they react with the platinum electrode, it is difficult to interprete the time course of the E_h' value in the complex medium used in our experiments.

We confirmed earlier observations (Leistner et al., 1980; Hechelmann and Leistner, 1984; Lücke, 1984) that the growth of facultatively anaerobic bacilli in canned sausage mixtures often stops at level below 10^6/g while the same organisms reach cell densities of 10^8/ml in the absence of oxygen when they are incubated in liquid cultures adjusted to similar pH, a_w and E_h' values, lactate and phosphate concentrations as present in the meat. In the absence of molecular oxygen, bacilli require fermentable carbohydrates for growth which are present only in minor amounts in meats and may not readily diffuse to the microcolonies of the bacteria, and they are sensitive to nitrite (Neumayr, 1983; Bell and De Lacy, 1982; compare Table I). As their spores are only moderately heat resistant and do not develop at temperatures below 10 °C, an appropriate heat treatment and proper chill storage are essential for the control of *B. subtilis* and *B. licheniformis* in canned meat products.

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