Volatile Compounds and Chemical Changes in Cured Pork: Role of Three Halotolerant Bacteria

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Of three bacteria strains isolated from Danish curing brines, only inoculated *Vibrio* sp. (168) brought about further change in chemical parameters and production of volatile compounds compared to uninoculated cured pork samples. *Vibrio* sp. (168) displayed nitrate reductase activity and caused the appearance of two characteristic volatile compounds, which were identified as 3-methylbutanal and 2-methylbutanal in the cured pork. The appearance of characteristic volatile compounds in cured pork inoculated with *Vibrio* sp. (168) coincided with the circumstance that *Vibrio* sp. (168) no longer could be detected by traditional plate count technique. The mechanism of formation of 3-methylbutanal and its possible significance in aroma development in cured meat products are discussed.

Keywords: *Cured pork, volatile compounds, 3-methylbutanal, halotolerant bacteria, Vibrio sp. (168)*

INTRODUCTION

The characteristic flavor of cured meat has been attributed to the use of sodium nitrite in the curing process (Ockerman *et al.*, 1973; Mottram *et al.*, 1984; Noel *et al.*, 1990). However, empiric observations have proven that introduction of new process technology in cured meat production gives rise to inferior flavor development (Anonymous, 1992). This is supported by a recent investigation which showed that a greater number of volatile compounds were developed in bacon produced according to traditional process technology compared to a bacon product prepared by new technology (Andersen and Hinrichsen, 1994). These observations strongly indicate that factors other than sodium chloride and sodium nitrite underlie cured flavor development in such products. The main difference between new and traditional process technology within bacon production is that the former leaves out the tank curing process, as this results in a considerable reduction in production time.

Cover brine used in the tank curing process of bacon has been produced by backsloppings in local slaghterhouses for decades. The result is a highly selected microflora in the cover brine (Leistner, 1959; Gardner, 1982). This microflora has been known for a long time to be important for color formation, stability (Leistner, 1958), and nitate reduction (Eddy and Kitchell, 1961; Meisel, 1988) in tank-cured meat products. Furthermore, sensory analysis of cured meat incubated with bacteria isolated from cover brines indicates better overall flavor development (Leistner, 1958; Petäjä *et al.*, 1972). However, no investigations are available which show that bacteria from cover brines produce specific aroma precursors or aroma compounds and how these may contribute to overall flavor development in cured meat.

The present study investigates the impact of three bacteria, *Halomonas elongata* (16), *Micrococcus roseus* (65), and *Vibrio* sp. (168) isolated from Danish cover brines, on chemical changes and production of volatile compounds in cured pork. Sterile pork cuts were inoculated with the respective bacteria strains, and changes in concentrations of NaCl, nitrate, and nitrite and in pH were followed over time, together with production of volatile compounds, as analyzed by headspace gas chromatography (HSGC). Headspace gas chromatography–mass spectrometry (HS-GC–MS) was used to identify volatile compounds that appeared due to inoculation of bacteria in cured meat samples. A hypothesis on the mechanism of formation of a bacterially produced compound is also proposed.

MATERIALS AND METHODS

Meat Samples. Deboned and derinded M. longissimus dorsi from pigs (Danish Grade A), 24-h post-mortem, were used as samples. Muscles were cooked by steam at 100 °C in an autoclave for 60 s, and the cooked meat layer was subsequently cut off under sterile conditions to produce aseptic meat samples. Each sterilized meat cut was subdivided into five pieces, packed in sterile plastic bags, and stored for no longer than 4 h at 4 °C for subsequent use.

Microorganisms. Three bacteria strains were isolated from Danish curing brines and tentatively characterized as *H. elongata* (16), *M. roseus* (65), and *Vibrio* sp. (168), as described elsewhere (Andersen and Hinrichsen, 1991). The isolates were grown with brain heart infusion medium (Difco) including 4% NaCl as substrate. Incubations were carried out at 20 °C for 4 days. Cells were harvested by centrifugation (11000*g*, 15 min) and resuspended in curing brine to an absorbance at 620 nm of 0.2 [approximately 10^7 colony forming units (CFU)/g].

Preparation of Cured Meat. Curing brine was prepared by autoclaving a solution of sodium chloride (21.02% w/w), sodium nitrite (0.13% w/w), and potassium nitrate (0.17% w/w).

Experiments were carried out in three series (one series for each bacteria strain). Sterile meat cuts (n = 25) were injected by multiple injection with sterile brine or brine with bacteria added. This resulted in an increase of 10% w/w. Samples were subsequently placed in sterile plastic bags, vacuum packed in polyethylene/aluminum bags, and stored at 20 °C.

Five samples from each group were taken out for chemical and microbial analyses after 2, 6, 8, 10, and 14 days of storage.

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Table 1. NaCl Concentration and pH in Cured Meat Samples Inoculated with *H. elongata* (16), *M. roseus* (65), or *Vibrio* sp. (168) and Respective Negative Controls during Storage for 2–14 Days at 20 °C

strain		bacteria					control				
		2 days	6 days	8 days	10 days	14 days	2 days	6 days	8 days	10 days	14 days
16	NaCl (%)	1.2	1.4	1.5	1.5	1.5	1.4	1.5	1.6	1.4	1.3
	SD[a]	0.18	0.05	0.09	0.06	0.10	0.10	*b*	0.10	0.09	0.19
	pH	5.5	5.6	5.6	5.7	5.8	5.5	5.6	5.5	5.7	5.8
	SD	0.01	0.04	0.02	0.03	0.01	0.02	*b*	0.06	0.03	0.04
65	NaCl (%)	1.5	1.5	1.5	1.6	1.7	1.4	1.5	1.4	1.5	1.5
	SD	0.06	0.08	0.05	0.02	0.18	0.07	0.11	0.08	0.16	0.01
	pH	5.6	5.7	5.6	5.7	5.6	5.6	5.7	5.6	5.6	5.7
	SD	0.06	0.06	0.03	0.03	0.05	0.07	0.08	0.06	0.03	0.01
168	NaCl (%)	1.7	1.6	1.6	1.6	1.5	1.6	1.5	1.6	1.7	1.7
	SD	0.16	0.10	0.10	0.05	0.00	0.07	0.06	0.15	0.07	0.04
	pH	5.4	5.6	5.8	5.6	5.7	5.6	5.5	5.7	5.5	5.8
	SD	0.09	0.04	0.04	0.04	0.03	0.17	0.00	0.06	0.05	0.03

[a] Standard deviation ($n = 5$). [b] Less than three registrations.

Chemical Analyses. Sodium chloride was determined by potentiometric titration with $AgNO_3$ in an autotitrator (Radiometer, Denmark), as described elsewhere (NMKL, 1974), and results are given as percent (w/w) NaCl.

Nitrate and nitrite were measured spectrophotometrically at 546 nm using the diazotization reaction of sulfanilamide and subsequent coupling with *N*-(1-naphthyl)ethylenediamine (NMKL, 1982). Results are given as parts per million of KNO_3 and $NaNO_2$.

A Knick Portamess Model 751 pH meter and a direct insertion probe electrode (Ingold Lot 406-M3) were used for pH measurement. pH is based on an average of three measurements in each sample.

Extraction of Volatile Compounds and Headspace Gas Chromatography. Three 2-mm slices of each meat sample (30 g) were cut into small pieces and placed in a 500-mL conical flask with a Dreschel head joined to a Tenax trap (Tenax TA mesh 60-80). One milliliter of a 2 ppm aqueous solution of heptyl chloride in a small cup was placed in the flask. The flask was sealed with parafilm and equilibrated for 30 min at 50 °C. Volatile compounds were purged onto a Tenax trap with ultrapure nitrogen for 10 min at a flow rate of 60 mL/min.

Volatile compounds were thermally desorbed from the Tenax trap (250 °C; 30 min) in a Spantech thermal desorber (TD4) and retrapped on a Tenax-packed cold trap maintained at −60 °C. Injection into the GC column was by thermal desorbtion of the trap at 300 °C for 59 s with a split of 1:10.

For GC analysis a Perkin-Elmer capillary gas chromatograph 8120, a data collection interface (PE Nelson 900), and a computer were used. A flame ionization detector (FID) held at 250 °C was used, while the injector temperature was 200 °C.

Chromatografic separations were performed with a DB-1701 capillary column (J&W Scientific; 30 m × 0.25 mm i.d.; film thickness 0.1 μm) using He as carrier gas (linear flow 29.17 cm/s). The oven temperature was held at 40 °C for 10 min and then raised from 40 to 250 °C at 6 °C/min with a final holding time of 5 min. Relative retention times (RRT) were calculated as the ratio between retention times of the unknown sample and heptyl chloride.

Headspace Gas Chromatography–Mass Spectrometry Analyses of Volatile Compounds. Samples for HSGC–MS were stored at −20 °C for no more than 12 weeks wrapped in aluminum foil and vacuum packed in a high oxygen barrier emballage. Extraction of volatiles and chromatographic separations were carried out as described above; however, a Varian gas chromatograph equipped with a Finnigan MAT ITS40 ion trap was used for GC analysis. The linear flow was 27.78 cm/s.

The mass spectra were obtained with electron impact ionization with autoion control (background mass 45, peak threshold 1, mass defect 0) in the range 15–250 amu. Only samples stored for 14 days at 20 °C were analyzed by HSGC–MS.

Bacterial Sampling. Total aerobic and anaerobic microbial load was determined for each sampling. After appropriate dilutions in 0.1% Peptone (Difco) including 4% NaCl (w/w), plate count agar with added 4% NaCl (PCA) was used as plating media for aerobic incubation and double-layer PCA was used as plating media for anaerobic incubation. Incubation was carried out for 5 days at 20 °C.

Statistical Analysis. Chemical data were tested for linearity and normal distribution and subsequently analyzed by analysis of variance using SAS statistical software (SAS, 1988). LSD_{95} (least significant difference) values are given where appropriate.

RESULTS

Chemical Analyses. To let the curing brine distribute in the meat samples, chemical analyses were not carried out until 2 days after injection of the curing brine. Table 1 shows NaCl concentration (% w/w) and pH in meat samples during storage. No significant difference in NaCl content and pH in meat samples was found with regard to injection of brine with and without respective bacteria strains. This indicates that the starting material was uniform. pH was found to increase slightly during storage due to autolytic processes in meat samples, as the presence of the respective bacteria strains was observed not to have any significant effect ($P > 0.05$).

Effects of inoculation with the respective bacteria strains on nitrate concentration in cured meat samples are shown in Figure 1. Neither *H. elongata* (16) nor *M. roseus* (65) had any significant effect ($P > 0.05$) on nitrate concentrations in cured meat samples during storage compared to control samples. However, inoculation of *Vibrio* sp. (168) in cured meat samples resulted in a significant decrease ($P < 0.001$) in the nitrate concentration during storage (Figure 1). Figure 2 shows change in nitrite concentration in cured meat samples during storage. A significant decrease ($P < 0.001$) in the nitrite concentration, which was independent of presence of bacteria, was observed in all meat samples during storage. However, in contrast to *H. elongata* (16) and *M. roseus* (65), the inoculation of *Vibrio* sp. (168) resulted in a significantly higher ($P < 0.001$) concentration of nitrite after 8 days of storage and throughout the storage period (Figure 2).

Headspace Gas Chromatography (HSGC) and Headspace Gas Chromatography–Mass Spectrometry

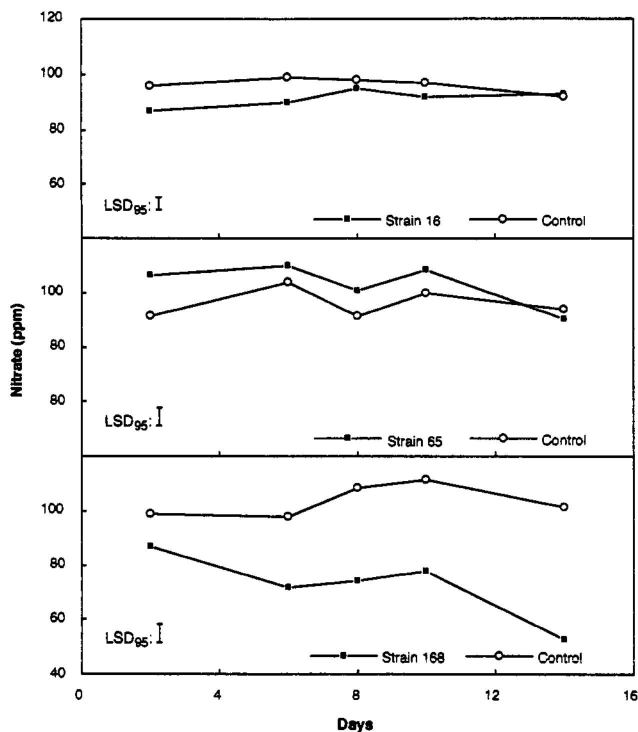


Figure 1. Changes in nitrate concentrations in meat with and without inoculation of *H. elongata* (16), *M. roseus* (65), or *Vibrio* sp. (168).

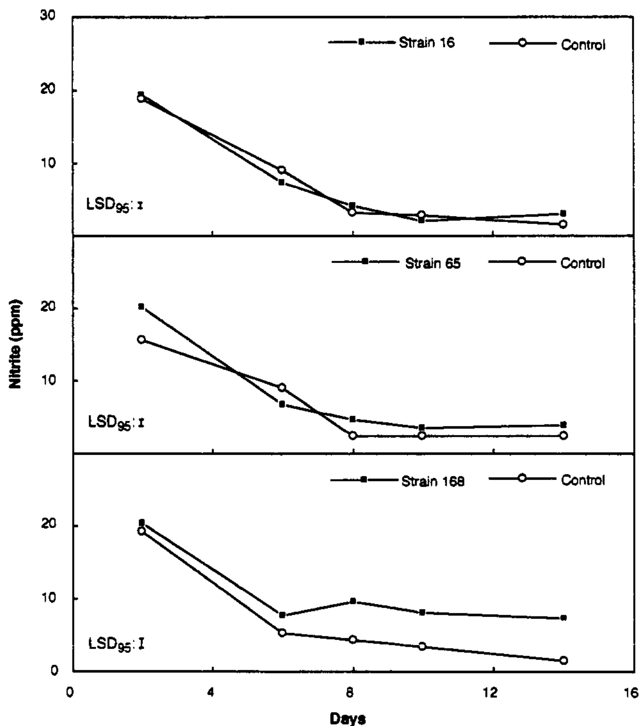


Figure 2. Changes in nitrite concentrations in meat with and without inoculation of *H. elongata* (16), *M. roseus* (65), or *Vibrio* sp. (168).

(HSGC–MS) Analyses. Representative gas chromatograms of volatile compounds in headspace from cured meat samples with and without inoculation of bacteria after 14 days of storage are shown in Figures 3–5. No differences in volatile compounds were observed between cured meat samples inoculated with *H. elongata* (16) and *M. roseus* (65) and their respective control samples (Figures 3 and 4). In contrast, inoculation with *Vibrio* sp. (168) resulted in the appearence of two volatile compounds, peaks 8 and 9 in the chromatogram, which did not appear in control samples, as seen in Figure 5. Results of subsequent HSGC–MS analysis of volatile compounds from cured meat samples are given in Table 2. This analysis identified compounds 8 and 9 as 3-methylbutanal and 2-methylbutanal, respectively, the latter only tentatively, as characteristic volatile compounds in those samples inoculated with *Vibrio* sp. (168).

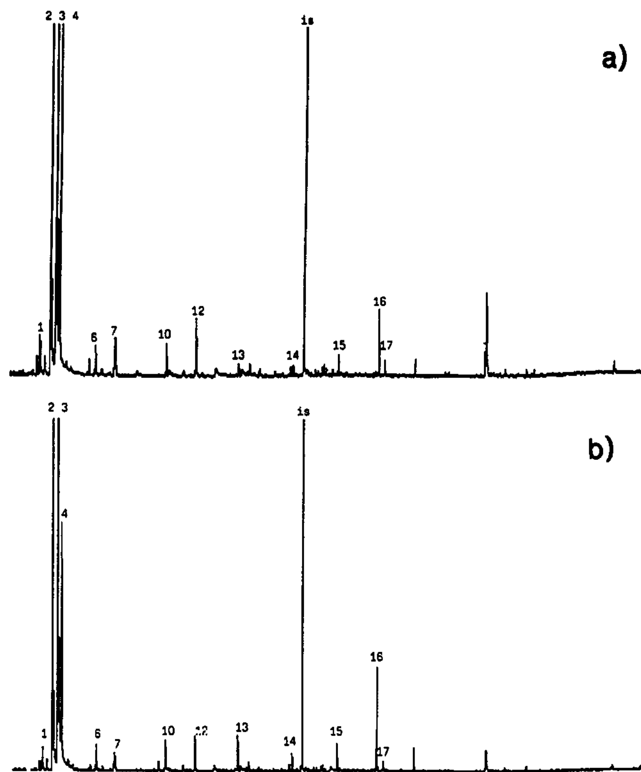


Figure 3. Representative gas chromatogram of volatile compounds in headspace from cured meat (a) with and (b) without (negative control) inoculation of *H. elongata* (16) after 14 days at 20 °C. is, internal standard (heptyl chloride).

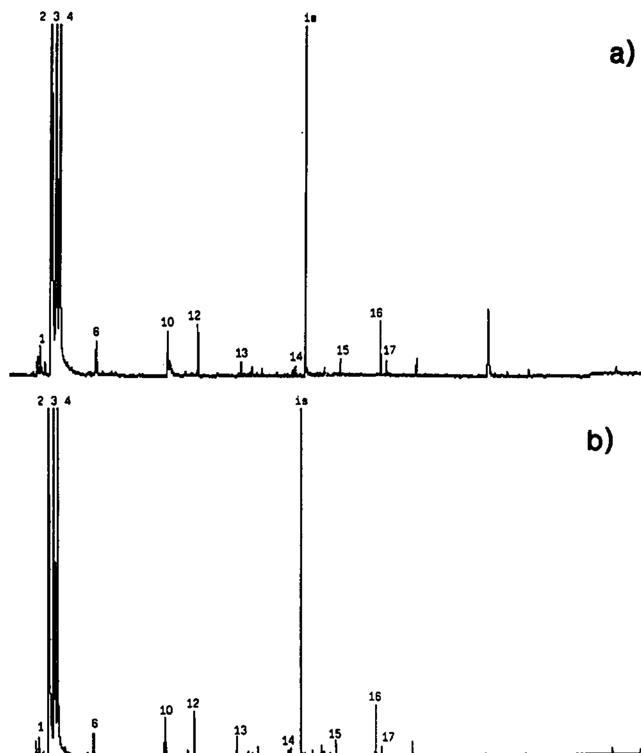


Figure 4. Representative gas chromatogram of volatile compounds in headspace from cured meat (a) with and (b) without (negative control) inoculation of *M. roseus* (65) after 14 days at 20 °C. is, internal standard (heptyl chloride).

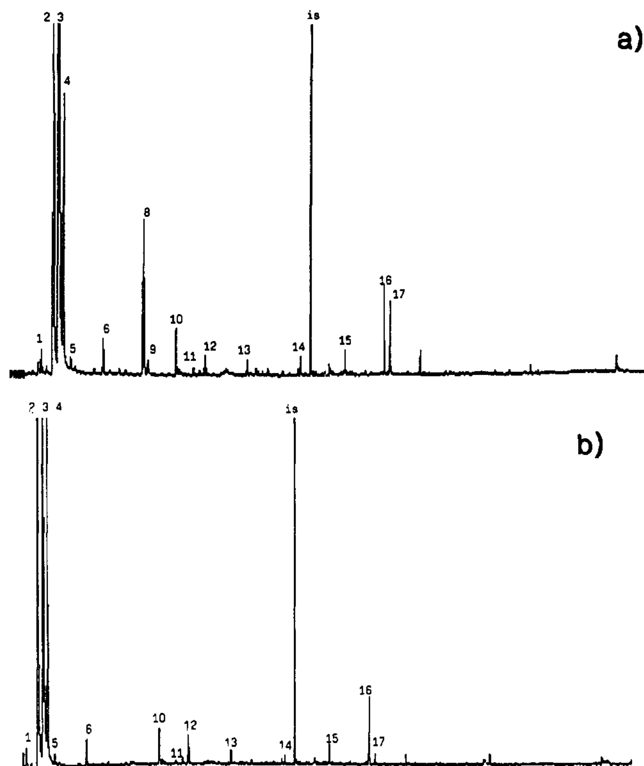


Figure 5. Representative gas chromatogram of volatile compounds in headspace from cured meat (a) with and (b) without (negative control) inoculation of *Vibrio* sp. (168) after 14 days at 20 °C. is, internal standard (heptyl chloride).

Table 2. Volatile Compounds Identified from Cured Pork Samples

peak no.	compound	ID[a]
1	acetaldehyde	RRT, MS
2	ethanol	RRT, MS
3	acetone	RRT, MS
4	2-propanol	RRT, MS
5	2-methylpropanal	MS
6	butan-2-one/diacetyl	RRT, MS
7	methyl branched alkane	MS
8	3-methylbutanal	RRT, MS
9	2-methylbutanal	MS
10	pentan-2-one	MS
11	acetic acid	RRT, MS
12	unknown	
13	3-methylbutanol	RRT, MS
14	propanoic acid	RRT, MS
15	contaminant	
16	contaminant	
17	contaminant	

[a] RRT, relative retention time compared to authentic compounds. MS compared to NIST library spectra and reference spectra.

Table 3. Semiquantitative Representation of 3-Methylbutanal and 2-Methylbutanal in Cured Meat Samples Inoculated with *Vibrio* sp. (168) during Storage

	storage time (days)				
	2	6	8	10	14
3-methylbutanal	–[a]	+	+	++	+++
2-methylbutanal	–	–	+	+	+

[a] –, not found; +, peak area 0–1500; ++, peak area 1500–3000; +++, peak area 3000–4500. Each quantitation is based on mean peak areas from five chromatograms. Peak area of 2 ppm of internal standard (heptyl chloride) corresponded to 11.000 ± 1.800.

Table 3 shows changes in concentrations of these two volatile compounds during storage of cured meat samples inoculated with *Vibrio* sp. (168). 3-Methylbutanal appeared after 6 days, while 2-methylbutanal appeared after 8 days. Only the former increased during the storage period, while the latter remained at the same level from day 8 and throughout the storage period.

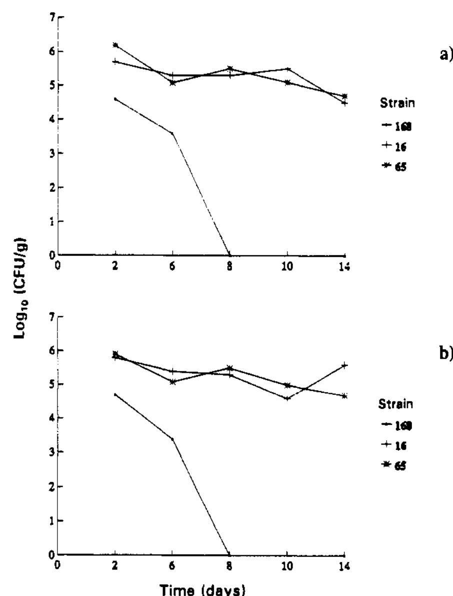


Figure 6. Aerobic (a) and anaerobic (b) plate counts [PCA + 4% (w/w) NaCl; 20 °C] during storage of cured meat samples inoculated with *H. elongata* (16), *M. roseus* (65), or *Vibrio* sp. (168).

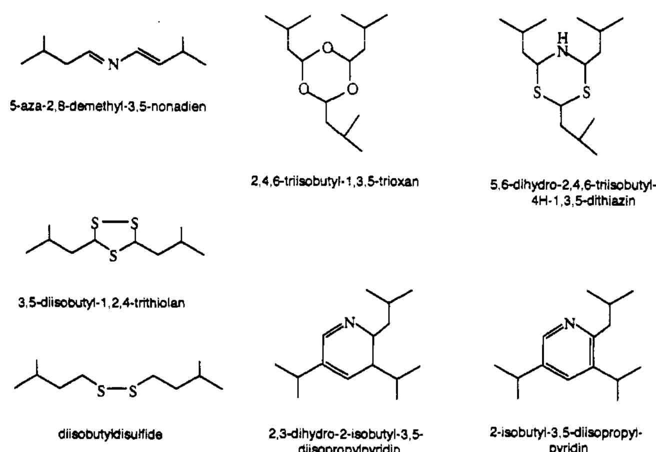


Figure 7. Complex reaction products identified in the reaction between 3-methylbutanal and ammonium sulfide according to Shu and co-workers (Shu and Mookherjee, 1985; Shu *et al.*, 1985).

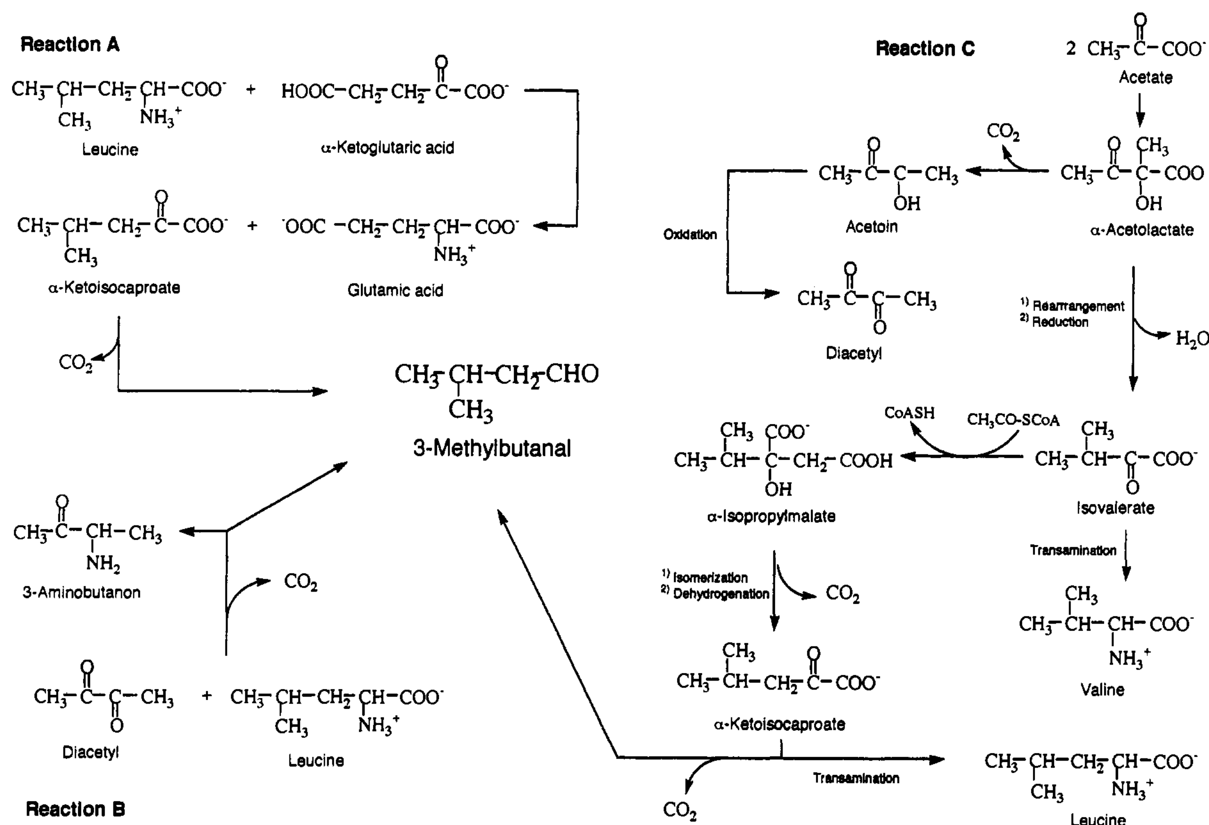
Microbial Analyses. Curing brines including *H. elongata* (16), *M. roseus* (65), and *Vibrio* sp. (168) had 50 × 10^7, 13 × 10^7, and 5 × 10^7 CFU/g, respectively, as determined by traditional plate count technique.

Aerobic and anaerobic plate counts during storage of cured meat samples inoculated with *H. elongata* (16), *M. roseus* (65), or *Vibrio* sp. (168) are shown in Figure 6. In general, anaerobic incubation resulted in slightly lower colony forming units per gram than aerobic incubation. However, the course during storage was equal in both circumstances. No increase in colony forming units per gram was observed in cured meat samples inoculated with any of the three examined bacteria strains during the storage period (Figure 6). *H. elongata* (16) and *M. roseus* (65) remained at the same level throughout the storage period, while the number of *Vibrio* sp. (168) decreased and became nondetectable after 8 days by traditional plate count technique.

DISCUSSION

Chemical analyses and headspace gas chromatography revealed that only *Vibrio* sp. (168) of the three halotolerant

Scheme 1



bacteria isolated from Danish cover brines had an impact on the cured pork model system.

Nitrite content in all cured meat samples decreased drastically during storage. We primarily assign this to the reaction between nitrite and meat proteins (nitrosation reactions) (Kanner *et al.*, 1992; Skibsted, 1992). After 6–8 days of storage, however, cured pork samples inoculated with *Vibrio* sp. (168) had a higher nitrite content compared to control samples. Compared to the continuous decrease in nitrate content in cured pork inoculated with *Vibrio* sp. (168) (Figure 1), this shows that *Vibrio* sp. (168) displays nitrate reductase activity in our meat system. A rough estimate of consumed nitrate in cured pork samples inoculated with *Vibrio* sp. (168) showed that only 50% was recovered as nitrite, which indicates that *Vibrio* sp. (168) subsequently may reduce nitrite to NO and N_2.

Parallel with the registered higher levels of nitrite in cured pork samples inoculated with *Vibrio* sp. (168) compared to control samples, two volatile compounds appeared. One was identified as 3-methylbutanal, while the other was tentatively identified as 2-methylbutanal. Appearance of these two volatile compounds and the registered nitrate reductase activity coincided with the fact that *Vibrio* sp. (168) could no longer be detected by traditional plate count technique (Figure 6). The registered effects of *Vibrio* sp. (168) could appear because of cell lysis or because cells enter a "nonculturable" stage, as descibed for *Vibrio* or *Vibrio*-like species by Gotschall (1992). Here, the fact that addition of disrupted *Vibrio* sp. (168) cells to cured meat samples did not show any effect (results not shown) indicates that the latter was the case. The nonculturable stage of *Vibrio* sp. takes place during limited nutrient supply (Gotschall, 1992). As the present meat system probably is equal to a limited nutrient supply situation, a nonculturable stage of inoculated *Vibrio* sp. (168) could be expected. Furthermore, the observed increase in metabolism of *Vibrio* sp. (168) in connection with a possible nonculturable stage is in agreement with results of Nyström and co-workers (Nyström *et al.*, 1989, 1990).

Both 3-methylbutanal and 2-methylbutanal have recently been found to be volatile compounds in traditionally cured bacon (Andersen and Hinrichsen, 1994) and dry-cured ham (Parma ham) (Barbieri *et al.*, 1992) and are often reported in other nitrite-cured meat products (Berdagué *et al.*, 1991; Golovnya *et al.*, 1982; Lillard and Ayres, 1969).

3-Methylbutanal has a powerful odor, which must be expected to influence the overall aroma in products, in which it is present. According to Shu and co-workers (Shu and Mookherjee, 1985; Shu *et al.*, 1985), 3-methylbutanal reacts at low temperature (~5 °C) with ammonium sulfide to form the compounds shown in Figure 7. 3,5-Diisobutyl-1,2,4-trithiolane (TT) and 5,6-dihydro-2,4,6-triisobutyl-4*H*-1,3,5-dithiazine (DT) are both claimed to display cured aroma (Shu *et al.*, 1985). As hydrogen sulfide and ammonia are both likely to be formed in meat and meat products (Hemme *et al.*, 1982; Gottschalk, 1985), these may react with the 3-methylbutanal present in cured meat products, giving rise to the butanedithiol and aminobutanethiol of 3-methylbutanal, which subsequently in the presence of hydrogen sulfide may react further and cause formation of TT and DT as shown by Shu *et al.* (1985). It is therefore likely that the presence of *Vibrio* sp. (168) is a decisive factor in connection with formation of overall flavor in tank-cured bacon products.

The branched-chain aldehyde 3-methylbutanal may arise from oxidative deamination–decarboxylation of leucine via Strecker degradation (Garcia *et al.*, 1991) (Scheme 1, reaction B). Alternatively, 3-methylbutanal can be produced as a byproduct during the biosynthesis of the amino acids valine and leucine (Belitz and Grosch, 1987). Under these circumstances 3-methylbutanal is formed by decarboxylation of α-ketoisocaproate, as shown in Scheme 1, reaction C (Belitz and Grosch, 1987). Finally,

3-methylbutanal may arise from the nonoxidative transamination–decarboxylation of leucine, as previously described for *Streptococcus lactis* var. *maltigenes* by MacLeod and Morgan (1955) and outlined in Scheme 1, reaction A.

Unexpected reactions might occur under extreme conditions, as found in cured pork meat. However, the fact that formation of 3-methylbutanal via Strecker degradation of leucine normally occurs at temperatures near 90 °C in alkaline media does not make this reaction pathway likely. Compared with the result that 3-methylbutanal only appeared in cured pork inoculated with *Vibrio* sp. (168) excludes the Strecker degradation of leucine as a possible source of 3-methylbutanal in cured pork meat. Neither of the two other stated mechanisms can be excluded as possible sources of 3-methylbutanal in cured pork meat inoculated with *Vibrio* sp. (168), according to our present knowledge. However, earlier observations that certain *Vibrio* sp. increase their intake of leucine during limited nutrient supply (Nyström *et al.*, 1986) may indicate that 3-methylbutanal is formed in a similar way, as found for *S. lactis* var. *maltigenes* (MacLeod and Morgan, 1955).

2-Methylbutanal can be formed as a byproduct during the biosynthesis of isoleucine (Belitz and Grosch, 1987). 2-Methylbutanal has an odor treshold value that is at least a magnitude higher than that of 3-methylbutanal (Belitz and Grosch, 1987). Presumably, this means that the presence of this compound will not contribute directly to the overall flavor of cured meat products; however, it cannot be excluded that 2-methylbutanal reacts with other compounds present and thereby contributes to the overall flavor of cured meat products.

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