Growth Profiles of *Vibrio* species Isolated from Danish Curing Brine

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SUMMARY:

Six different *Vibrio* species have been isolated from five commercial Danish curing brines used in the production of tank cured bacon sides. The six species were identified as *V. proteolyticus, V. nereis, V. costicola, V. logei, V. vulnificus* and *V. alginolyticus*, respectively, within *approx.* 75 % homology. Addditional, growth of isolated species were followed as a function of temperature (2, 5, 8, 12 C), pH (6.0, 5.8, 5.5, 5.2, 4.9, 4.6) or NaCl-concentration (0.5, 8, 12, 16, 20, 24 %) in a BHI-medium (Brain-Heart-Infusion) using a mirotiter-plate technique. These results showed in contrast to normal nutritional characterization (criteria of *Bergey's Manual*) a further differentiation of some of the isolated *Vibrio* species. The growth profiles as a function of pH and to a minor ewtent the growth profiles as a function of salt concentration, respectively, indicate that at least some of the isolated species could be differentiated further into two separate groups. Especially *V. proteolyticus* and *V. nereis*, which were the dominating *Vibrio* species found with present isolation technique , were seperated in two distinct groups. One group, which could grow at pH > 5.8, and another group, which could grow down to pH 4.9.

INTRODUCTION:

It is debatable whether bacteria in and on bacon are benificial. Flavour production in this product is postulated to be a result of protein changes induced by bacteria (Jones, 1949), and it is stated (Petäjä et al., 1972) that old curing brine is added to fresh brine so that the useful organisms in the old brine are transferred to the new one in order to ensure quality of the product. However Jones (1949) too indicated that due to the proteolytic activity of many of the organisms found in curing brines, they could also produce off-odours and even soft spots in the skin when present in high numbers. This is in agreement with the results of Gardner (1973, 1975), who found that a high level of bacteria in curing brines (cover brines) resulted in poor maturation conditions for bacon products, and he also found that a high total counts correlate with the number of vibrios present in curing brines. *Vibrio* have for a long time been stated as an important spoilage organism in bacon (Smith, 1938; Gibbons & Rose, 1950; Gardner, 1971,1973, 1975) and especially when the curing process has been out of control e.i. pH > 5.9 (Gibbons & Rose, 1950; Gardner, 1971) and temperatures > 7 °C (Gardner, 1973). Contrary to these results Petäjä et al. (1973) found that a *Vibrio sp.*, identified as a *Vibrio costicola*, added to a brine for tank cured ham lead to a superior product as regards all the properties examined (colour of cut surface, texture, flavour) to the control after the products was air-dried. In wiev of the fact that *Vibrio costicola* and *Vibrio costicola* sub.sp. *liquefaciens* recently have been postulated to be associated as a possibly spoilage bacteria in cured meat (Gardner, 1980-81), we have found it essential for future quality ensurance of bacon processing to elucidate, if presence of different *Vibrio* sp. in curing brines are beneficial or not for maturation of bacon products. The aim of the work described in this paper was preliminaryly to isolate, identify and define the growth areas, respectively, of different *Vibrio* sp. from Danish curing brines, which are going to be tested for their role in the maturation process of cured bacon.

MATERIALS & METHODS:

Examination of brines and isolation of micro-organisms. Five curing brines (cover brines) from different Danish slaughterhouses, with the total bacterial count and chemical composition shown in Table 1, were chosen as sampling media. There were withdrawed 1 ml samples from the different brines and appropriate tenfold dilutions in 0.1 % peptone water plus 20 % NaCl (w/v) were made. Subsequently, 1 ml from each dilution was used to inoculate nutrient agar (NA) plates (Difco) including either 4 % or 15% NaCl, which were then incubated at 4 °C and 20 °C, respectively.

Colonies (166) picked according to their different colony morphology (to get most of species represented in the brines) from the

lowest countable dilution were tested for cell morphology, KOH-test (Gregersen, 1978), catalase production, oxidase reaction and capability of glucose fermentation (Hugh & Leifson, 1953).

Strains (63) found to be Gram-negative, catalase positive, oxidase positive and capable to ferment glucose were subsequently tested for sensibility to vibriostaticum (0/129) to elucidate possible presence of *Halomonas elongata*. This resulted in 39 strains, which were maintained in brain heart infusion medium included 4 % NaCl.

Table 1. Bacteriological and chemical analyses of curing brines.

	Brine 1	Brine 2	Brine 3	Brine 4	Brine 5
Total count[a]	$4.2*10^5$	$5.8*10^6$	$1.2*10^6$	$3.4*10^6$	$2.7*10^5$
pH	6.12	6.10	6.10	6.08	6.05
KNO_3 (g/l)	2.4	1.9	2.4	2.4	2.4
$NaNO_2$ (g/l)	1.2	0.9	1.2	1.2	1.2
NaCl (g/l)	260	210	271	270	266
°Bé	23.3	19.3	23.3	23.1	22.8

[a]Incubation conditions; Nutrient agar incl. 4% NaCl, 20°C 6 days.

Identification of strains: The classification given in Bergey's Manual (Baumann et al., 1984) was followed. Strains were identified with a strain type when the similarity was approx. 75 %. Following tests have been carried out for further characterization of strains identified as *Vibrio* sp.: Oxidase, sucrose, D-galactose, cellobiose, D-mannitol, lactose, L-arginine, L-ornithine, putrescine, gelatinase, γ-aminobutyrate, gas production from glucose fermentation, growth in 10% NaCl and nitrate reductase activity.

Preparation of microtiter test plates for growth profiles. Substrate chosen for use in determination of growth profiles of *Vibrio* strains was brain heart infusion medium (BHI, Merck). For pH-profilation was added NaH_2PO_4 and NaCl to give a bufferstrength of 100 mM and a salt content of 8 %. For NaCl-profilation media were included 0.5, 8, 12, 16, 10 and 24 % NaCl and for temperature-profilation medium was adjusted to 8 % NaCl both with pH of 7.2. Subsequently were all media heat-sterilized and pH were adjusted in media for pH-profilation (4.6, 4.9, 5.2, 5.5, 5.8 and 6.0). An 5-channel multi pipettor was used to add 200 µl of the media to wells of sterile 96-well polystyrene microtiter-plates with covers, and subsequently was added 25 µl of isolated strains in the late log-phase. Plates were then wrapped in plastic film and stored at 20 °C except plates for temperature-profilation, which were stored at 2, 5, 8, and 12 °C, respectively. Plates were subsequently read at an automated microtiter-plate reader (Biorad), which measured optical density at 620 nm. This last procedure was repeated every day over a periode of ten days.

Statistical test. Growth of the *Vibrio* strains in micotiter test plates was analyzed using a students t-test on five replicates of each combination, and growth was defined when different between controls and the five replicates was significant different at the 99 % level.

RESULTS AND DISCUSSION:

A tentatively identitification based on approx. 75 % homology with fourteen characteristica for identification of *Vibrio* ssp. according to Bergeys Manual resulted in an identification of six species of *Vibrio*: *V. proteolyticus* (35.9%), *V. nereis* (35.9%), *V. costicola* (10.3), *V. logei* (10.3%), *V. vulnificus* (5.1%) and *V. alginolyticus* (2.5%). Non of the isolated strains showed all

Table 2. Characteristics of strains of *Vibrio* isolated.

Vibrio. sp.	*proteolyticus*	*nereis*	*costicula*	*logei*	*vulnificus*	*alginolyticus*
No. of strains	(14)	(14)	(4)	(4)	(2)	(1)
Oxidase	100% (+)	100% (+)	100% (+)	100% (+)	100% (+)	100% (+)
Sucrose	100% (-)	0% (+)	0% (+)	100% (-)	100% (-)	0% (+)
D-Galactose	86% (-)	100% (-)	100% (-)	100% (+)	100% (+)	0% (d)
Celloblose	100% (-)	100% (-)	75% (-)	0% (+)	50% (+)	100% (-)
D-Mannitol	36% (+)	100% (+)	100% (+)	100% (+)	100% (+)	100% (+)
Lactose	100% (-)	100% (-)	100% (-)	50% (-)	50% (+)	100% (-)
L-Arginine	79% (+)	100% (+)	100% (-)	100% (-)	100 % (-)	100% (+)
L-Ornithine	79% (+)	100% (+)	100% (-)	100% (-)	100% (-)	100% (-)
Putrescine	71% (+)	43% (+)	50% (-)	100% (-)	100% (-)	0% (d)
Gelatinase	30% (+)	57% (d)	100% (-)	100% (-)	50% (+)	0% (+)
γ-Aminobutyrate	79 % (-)	100% (-)	50% (-)	100% (-)	100% (-)	100% (-)
Gas from glucose	100% (-)	100% (-)	100% (-)	100% (-)	100% (-)	100% (-)
Growth in 10% NaCl	100%	100%	100%	100%	100%	100%
NO_3^- - NO_2^-	71% (+)	86% (+)	100% (d)	100% (+)	100% (+)	100% (+)

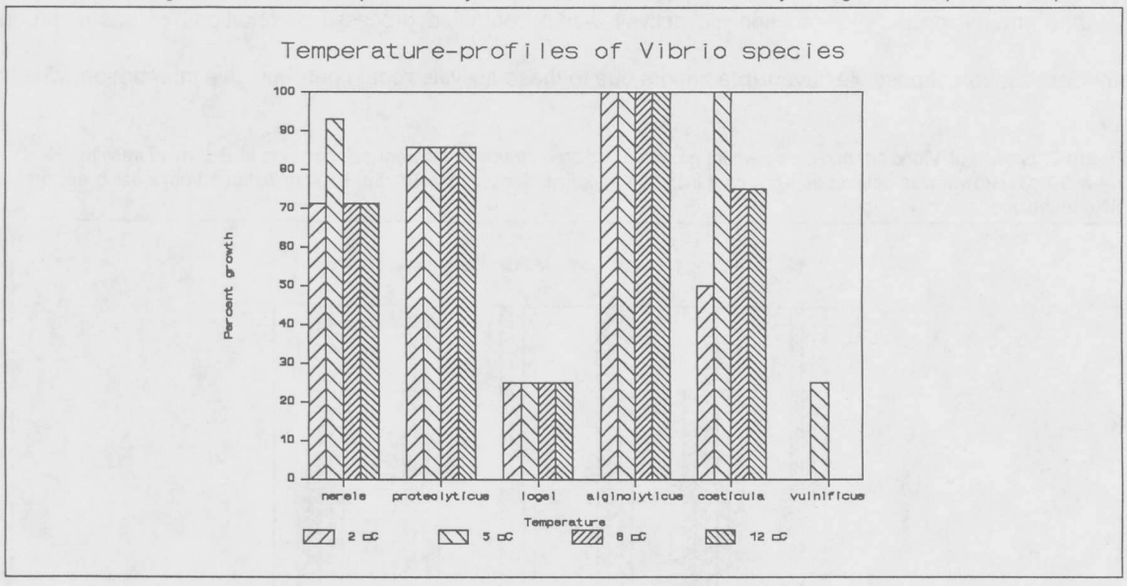
characteristics described for relevant standard strains, which was to be expected according to the extreme enviroment from which strains were isolated. The characteristics of the *Vibrio* are shown in Table 2.

In an attempt fo further delineate isolated strains we stated that growth-profiles was a possible tool to give an alternative characterisation of each individual species according to their normal extreme living conditions, as this too could indicate under which conditions these organisms could be active during processing, maturation and storage of tank cured bacon types.

Growth-profiles of isolated strains as function of temperature, pH and NaCl in BHI-medium using microtiter-plate technique are seen in Fig. 1, 2 and 3, respectively.

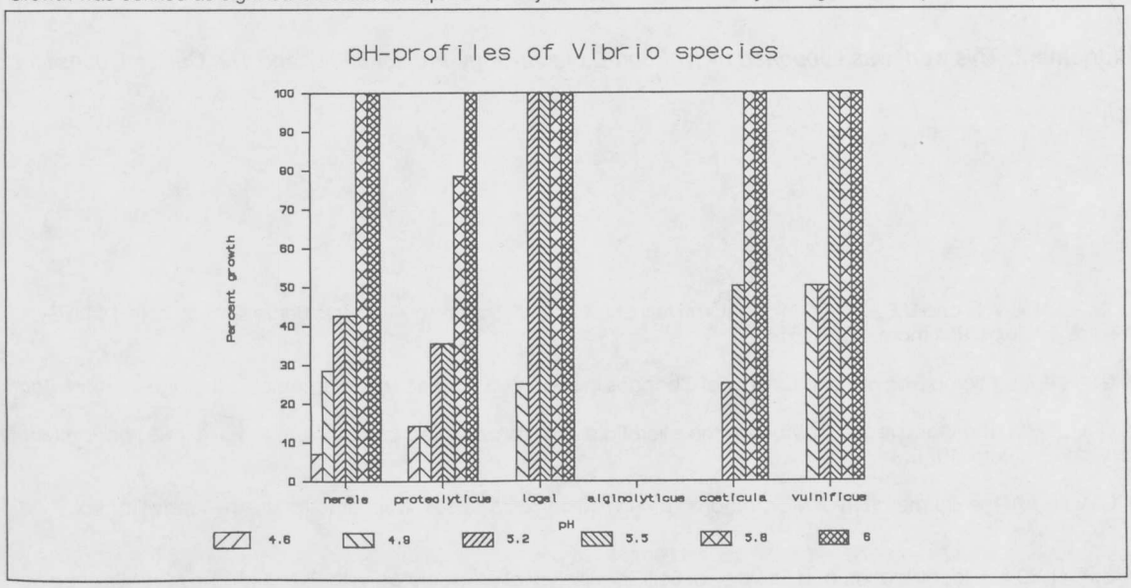
Temperature-profiles showed that most of the isolated strains were able to grow over the range tested, and only strains within the species *V. logei* and *V. vulnificus* were retarded in growth within temperatures tested. Regarding pH-profiles of isolated strains, these showed separation of almost all characterized species into two groups, one group, which could grow at pH > 5.8, and another group, which could grow down to pH 4.9. Only the strain characterized as *V. alginolyticus* was not able to growth in the pH-area tested. Finally showed NaCl-profiles that all isolated strains were able to grow at least to a salt-content of 12 %, and even at 24 % salt some of strains identified as *V. proteolyticus* were able to growth.

Figure 1. Growth of *Vibrio* sp. given as percent growth at different temperatures in BHI-medium plus 8 % NaCl at pH 7.2. Growth was defined as significant increase in optical density measured at 620 nm within 10 days using microtiter-plate technique.



Further analysis of observated growth-profiles of isolated *Vibrio* strains indicate that these organisms can be active during both processing and storage of tank cured bacon types, as normally processing and storage conditions for tank cured bacon are within the temperature-, pH- and NaCl-content ranges tested. Indeed these results do not give an answer to whether *Vibrio* sp.

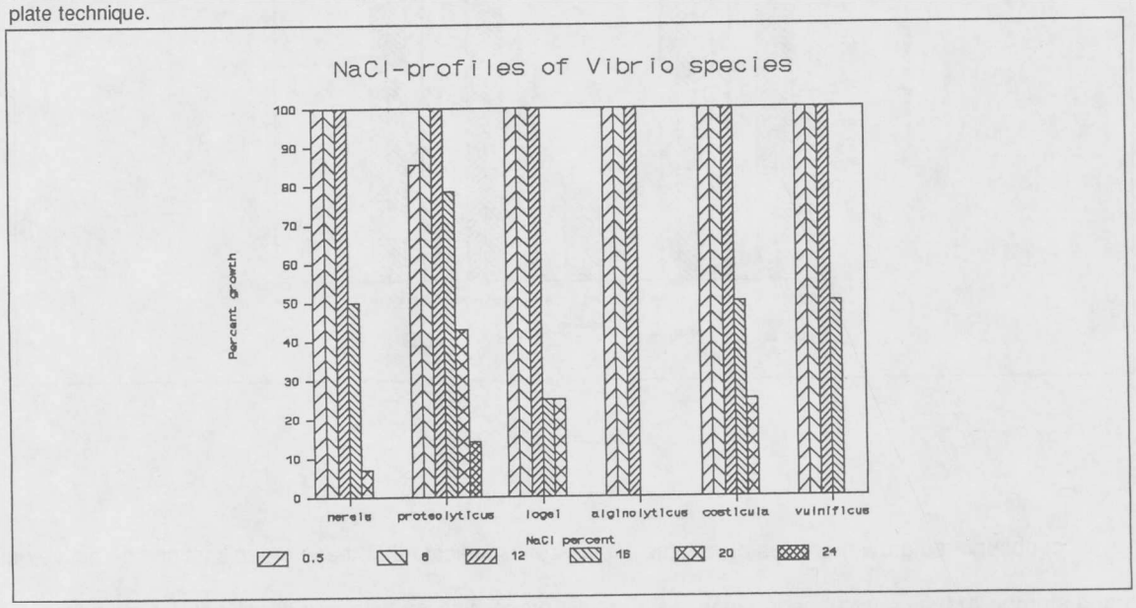
Figure 2. Growth of *Vibrio* sp. given as percent growth of isolated strains at different pH in BHI-medium plus 8 % NaCl at 20 °C. Growth was defined as significant increase in optical density at 620 nm within 10 days using microtiter-plate technique.



are benificial or not for maturation of tank cured bacon products, but they may indicate that *Vibrio* strains beneficial in maturation of tank cured bacon are different from *Vibrio* strains, which deteriorate the products. This is based on the fact that *Vibrio* especially is considered as a spoilage organism, when the curing process is out of controll e.i. pH > 5.9 (Gibbons & Rose,1950; Gardner, 1971), while *Vibrio* sp. normally not are considered as spoilage organisms, when the curing process is under controll

e.i. pH < 5.8, and compared with results from pH-profiles of isolated *Vibrio* strains in the present study. Additional investigations of isolated *Vibrio* strains' proteolytic and lipolytic activity within controlled processing conditions of bacon products, and subsequently identification of possible flavour-precursors due to these activities could perhaps give information, whether *Vibrio*

Figure 3. Growth of *Vibrio* sp. given as percent growth of isolated strains at different salt contents in BHI-medium with pH 7.2 at 20 °C. Growth was defined as significant increase in optical density measured at 620 nm within 10 days using microtiter-plate technique.



strains capable to growth beneath pH 5.8 are benificial in maturation of bacon products with respect to flavour production, while *Vibrio* strains only capable to growth at pH > 5.9 are to be considered as spoilage organisms in tank cured bacon products.

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