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# EFFECTS OF BALTIC FISHES FRESHNESS ON THERMAL RESISTANCE OF BACTERIAL SPORES

## II. EFFECT OF ICE COD FRESHNESS UPON HEAT RESISTANCE OF PUTREFACTIVE ANAEROBE P.A. 3679 SPORES SUSPENDED IN THE RAW AND PRE-COOKED FISH MINCE

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Key words: putrefactive anaerobe, thermal resistance, iced cod, fish mince.

Baltic cod from Dziwnów fishing grounds was stored in ice at 0° to 4°C up to 12 days after the catch. Deterioration of freshness was represented by fish minces prepared from stored fish at 3-day intervals, and next they were used as suspending media for testing the heat resistance of P.A. 3679 spores. The investigations proved that deteriorating freshness of raw material lowers thermal resistance of spores in minces that had been initially denaturated by heat (the drop of $D_{110.1}$ from 4.33 to 2.41 min. after 12 days). In the raw mince, where spores showed a much higher thermal resistance ($D_{110.1}$ = 5.74-6.62) the dependence was poorly marked.

The percentage of stable samples revealed a constant dependence on $\log_{10}$ of the initial number of spores (cans at various levels of intensity of thermal treatment).

## INTRODUCTION

The fundamental requirement for thermal processing of low-acid food is to obtain a high degree of reduction of the initial load of highly resistant to heat pathogenic and spoilage bacterial spores present in raw and semi-prepared products.

There is a certain view that thermal resistance of these bacterial spores is particularly dependent on physico-chemical properties of environment in which they are exposed to lethal effects of heat. Change-

ability of thermal inactivation is perceivable also in *Bacillus subtilis* populations which show moderate thermal resistance heated in extracts from Baltic fishes of varying freshness [17]. Yet in order to make more conspicuous the changes' in the physico-chemical properties of fish meat due to degrading freshness [12, 13] it seems that application of highly thermoresistant spores should be in order.

## MATERIALS AND METHODS

### 1. MATERIALS

Lyophilized culture of putrefactive anaerobe P.A. 3679 was obtained from American Type Culture Collection (Rockville, Maryland, USA).

Non-particle medium [5] was used for active inoculum preparation, and bi-phasic Annelis and Rowley's medium [3] for production of spores.

Cod used in the experiments originated from Baltic fishing grounds of Dziwnów vicinity, and was landed in April and May, 1975.

Cylindrical, $\phi$ 73×18 mm lacqured cans were used as standard containers for heat resistance tests.

### 2. PRODUCTION OF SPORES

Culture of the test organism has been initiated by transferring 0.1 ml of stock culture in liver broth to non-particle growth medium. After heat shock of 20 min at 80°C this specimen was incubated 8 hrs at 37°C. Two next screenings into fresh medium were made at 4 hrs intervals of incubation time at 37°C. The transferred quantities amounted always one tenth of fresh medium volume.

Volume of 1500 ml of sporulation medium was next inoculated with 50 ml of active culture, its proportion of liquid and solid phase was 1:2. This culture was incubated 6 days at 37°C which resulted in 90% sporulation.

Spores were subjected to repeated centrifuging at 1 000 g, and washings with ice-cold sterile distilled water in centrifuge typ T 23 (Janetzki, GDR). Finally, spores were resuspended in 100 ml of sterile distilled water in a flask to which several of glass beads were added. Stock spore suspension was stored at 4°C until used. It contained $1.5\times10^8$ of refractile spores per ml.

### 3. PREPARATION OF THE TEST SPORE LOADS

Test spore loads were prepared by depositing appropriate amounts of spore suspension dilutions onto central part of sterile 5×45 mm stripes of Whatman No. 3 filter paper [7]. Hamilton's micro-syringe was used for this purpose. Loads of $10^2$, $10^3$, $10^4$, $10^5$ and $10^6$ P.A. 3679 spores per stripe were prepared.

#### 4. PREPARATION OF SPORE INOCULATED CANS

Fishes were stored in ice at 0-4°C. Out of stored stock specimens were taken at 3-days intervals. Fishes were washed and filleted, and after skinning out, the meat was minced. Originating from gradually extended periods of storage e.g., 0, 3, 6, 9 and 12 days, they represented decreasing freshness of stored fish and were used as media for the heating of test spore loads. Minces representing all periods of storage were used in raw as well as heat denaturated form. Pre-cooking procedure was standardized e.g. 400 g portions of minces were heated at 110°C in "twist-off" glass jars to get lethal value of treatment equal $F_0$ of 0.1 min. These conditions reflect the effect of the initial heat treatment on the meat tissues of fishes in the course of canning. After tight filling of standard mince portion into test can, straightened spore strip was placed in its central part so that spore load das approximately in a geometrical center of a container, not touching its falls. After that, cans were hermetically sealed on laboratory closing machine. For comparison purposes out of both kinds of minces, control cans, not containing spores, were prepared.

#### 5. CONTROLLED HEAT TREATMENT OF INOCULATED CANS

Heat treatment of inoculated cans was carried out at 110°C ±0.3 in the steam retort (adapted horizontal autoclave type A-7, SMS Warsaw) under conditions which simulated commercial sterilization.

The course of each process was registered by electric recording thermometer type ZC-9 (Ellab, Denmark), equipped wih applicators of TCOSG type for retort temperature measurement, and TCK-33 needle type for the heat penetration measurement in a geometrical center of can.

The first stage of experiments comprised 25 processes of 24 cans containing raw mince in each process (5 concentrations of spores + control samples). In the second stage there were 10 processes with 36 cans of pre-cooked fish mince per process, representing two periods of storage (3 concentrations of spores + control samples). The third stage comprised 25 processes with 24 cans per process, one half with a non-denaturated mince, and second with pre-cooked mince from the same period of storage (concentration of spores $10^6$ per can).

#### 6. RECOVERY OF SPORES AFTER HEATING

Processed cans were incubated at 37°C without screening onto recovery media. Cans showing hard swells with liquefaction of contents were counted as positive samples. Incubation was carried out up to 700 days, with daily control for swells up to 30 days, and next after each 3-5 days.

The variable conditions were as follows: freshness of stored fish, being function of storage time, number of spores per container, gradually

extended time of heating, use of two kinds of fish minces, originating from identical periods of storage e.g. raw mince and precooked mince.

Directly from the experiments the following data were collected: heat penetration curves, number of positive (swollen) cans after 700 days of incubation, incubation time required for detection of spoiled cans. The following parameters could have been calculated from these data: corrected time of heat treatment at 110,1°C-$t_{110.1}$ (min.) and lethal values — $F_0$ (min.) of processes, decimal reduction time — $D_{110.1}$ (min.) shown in particular experimental runs, and resultant percentage of microbial stability on the different levels of heat treatment — $p_s$ (%).

### 7. EVALUATION OF HEATING TIME CORRECTIONS AND LETHAL VALUES OF HEAT PROCESSES

Calculations of corrected heating times were made with assumption of z = 10°C [11, 16], with use of the analytical method [14] on the basis of heat penetration data. In these calculations Ball's equation [4] was applied in a form:

$$t_{110.1} = \int_0^t L' dt \text{ (min.)}$$

where,

$$L' = 10^{\frac{T-110.1}{10}}$$

L' — Rate of lethality at 110.1°C reference temperature.
T — Temperature measured in the geometric centre of the can corresponding to a given heating period; °C.
$t_{110.1}$ — Heating time at 110.1°C needed to reduce 90% of spore load; min.

Lethal values of these processes at 121.1°C — $F_0$ (min.) were calculated out of the same set of data [4, 14].

Stumbo's equation [16] was used for calculations of the decimal reduction times at 110.1°C, where:

$$D_{110.1} = \frac{t_{110.1}}{\log a - \log b}$$

a — number of spores before heating
b — number of spores after heating in time $t_{110.1}$ min.

## RESULTS

The results obtained for the raw minces were compared on the four levels of lethal values e.g. for $F_0$ amounting 0.86-1.50, 1.58-2.0, 2.04-2.97, and 3.10-4.03. They were not arranged accordingly to the period of storage but depending upon number of spores initially present in test cans e.g.

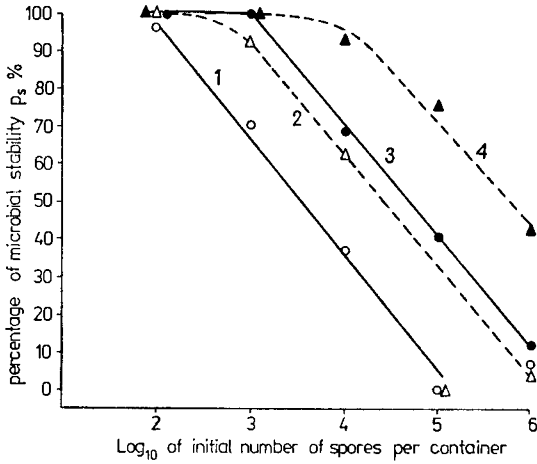


Fig. 1. Effect of number of P.A. 3679 spores introduced to raw cod mince, and severity of heat treatment upon microbial stability of processed cans; 1 — $F_o$ = 0.86-1.50 min.; N = 31; $n_o$ = 126; $n_s$ = 51; 2 — $F_o$ = 1.58-2.00 min.; N = 36, $n_o$ = 164; $n_s$ = 64; 3 — $F_o$ = 2.04-2.97 min.; N = 46; $n_o$ = 202; $n_s$ = 108; 4 — $F_o$ = 3.10-4.03 min.; N = 30; $n_o$ = 156; $n_s$ = 95. $F_o$ — equivalent of heating at 121.1°C (min.) calculated for z = 10°C; N — number of heat processes; $n_o$ — number of heated cans; $n_s$ — number of microbiologically stable cans

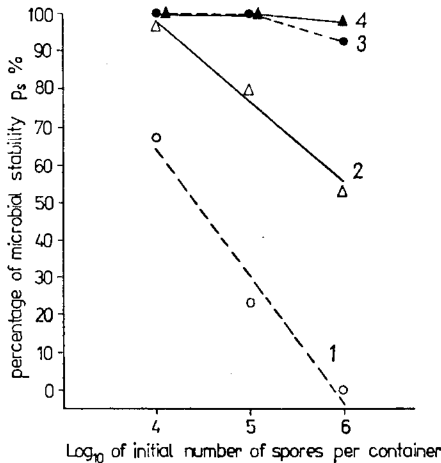


Fig. 2. Effect of number of P.A. 3679 spores introduced to precooked cod mince, and severity of heat treatment upon microbial stability of processed cans; 1 — $F_o$ = 0.97-1.02 min.; N = 15; $n_o$ = 90; $n_s$ = 27; 2 — $F_o$ = 1.58-1.73 min.; N = 21; $n_o$ = 134; $n_s$ = 93; 3 — $F_o$ = 2.00-2.74 min.; N = 22; $n_o$ = 135; $n_s$ = 129; 4 — $F_o$ = 2.80-4.03 min.; N = 41; $n_o$ = 260; $n_s$ = 257. $F_o$ — equivalent of heating at 121.1°C (min.) calculated for z = 10°C; N — number of heat processed; $n_o$ — number of heated cans; $n_s$ — number of microbiologically stable cans

$10^2$, $10^3$, $10^4$, $10^5$ and $10^6$ spores per container. Results of testing of 648 cans were collected in the mentioned order. Percent of microbial stability ($p_s$) was computed from the proportion of stable cans number ($n_s$) to the number of heated cans ($n_0$), representing defined number of spores per can and heat treatment severity. Calculated $p_s$ values were plotted against $\log_{10}$ values of the initial spore number per can (Fig. 1). The curves drawn through the plotted points show linearity in the range of $p_s$ 0-95%, and are parallel for the all levels of the thermal treatment severity.

It could be noted that increasing of the heat treatment severity, results in parallel shifting of appropriate curves in the direction of the increasing numbers of spores per can. It could be stead in analytical categories that independently of heat processing severity, the 10-fold rise of initial number of spores per container causes 30% decrease in number of microbiologically stable cans.

Results of testing of 619 samples of the pre-cooked cod minces showed similar pattern, and they were shown on Fig. 2. This comparison was made for three levels of spore contamination per container ($10^4$, $10^5$ and $10^6$) on the four levels of heat treatment severity — $F_0$ (min) e.g. 0.97-1.02, 1.58-1.73, 2.00-2.74 and 2.80-4.03. The above mentioned linearity of the $p_s$-curves is observed in this case only for two milder levels of the heat processing. So, when $F_0 = 0.97$-$1.02$ min., 10-fold rise of initial spore load gives 34% decrease of $p_s$ value, while for $F_0 = 1.58$-$1.73$ it causes 22% decrease of $p_s$ value.

The comparison of data presented on Fig. 1 and Fig. 2 shows that effectiveness of heat processing is much higher in the pre-cooked minces, and comparing to raw minces it could be proved by higher $p_s$ values obtained on the similar levels of process lethal values.

Table. Effect of decreasing freshness upon decimal reduction time*) $D_{110.1}$ of putrefactive anaerobe P.A. 3679 spores heated in cad minces

| Kind of cod mince | Period of storage at 0-4°C (days) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | 3 | | 6 | | 9 | | 12 | |
| | N | $D_{\bar{x}}$ (min) | N | $D_{\bar{x}}$ (min) | N | $D_{\bar{x}}$ (min) | N | $D_{\bar{x}}$— (min) | N | $D_{\bar{x}}$ (min) |
| Raw mince | 5 | 5.74 | 9 | 6.62 | 8 | 6.43 | 9 | 5.53 | 9 | 5.60 |
| | 3 | 4.60 | 6 | 5.10 | 6 | 5.50 | 4 | 5.60 | 5 | 5.60 |
| | | | | | 1 | 6.50 | 2 | 7.30 | 1 | 7.60 |
| Pre-cooked mince | 7 | 4.33 | 7 | 4.26 | 7 | 3.79 | 3 | 2.79 | 2 | 2.40 |
| | 3 | 2.70 | 3 | 2.70 | 1 | 2.10 | 1 | 2.00 | 1 | 2.00 |
| | 4 | 4.50 | 5 | 6.70 | 2 | 5.50 | 8 | 4.20 | 8 | 4.00 |

*) — evaluation of $D_{110.1}$ values was based upon results of heating of $10^4$-$10^6$ spores per container;
N — number of heat processes.

Comparison of thermal resistance of P.A. 3679 spores heated in fish minces representing gradually decreasing freshness is shown in Table 1. It has been made by calculation of decimal reduction times — $D_{110.1}$ (min.), based on Stumbo's thesis [16] that in final points of survival under defined heat treatment, the spoilage of cans is a result of out-growth from individual spores surviving treatment.

Besides $D_{110.1}$ values calculated on this basis, in Table 1 were listed approximate $D_{110.1}$ values — one set calculated from mild processes which did not yield reduction of positive samples (actual $D_{110.1}$ value higher from approximated value), and second set, calculated from processes which yielded sterility of all heated samples (actual $D_{110.1}$ value lower from approximated). With application of this procedure was possible to make an additional verification of the range which should enfold $D_{110.1}$ values calculated from partial spoilage data. As could be seen in Table 1, mean $D_{110.1}$ values being a measure of the freshness influence upon heat resistance of spores heated in fish minces, are falling in these limits. The only slight anomaly was found for the raw mince from 9-days storage period where $D_{110.1}$ value is slightly lower from down limiting value. In the raw minces out of 3 and 6 days of storage, the marked increase of the heat resistance could be noted, while in the minces representing 9- and 12-days period of storage, $D_{110.1}$ values were approximately equal with those determined in the fresh raw fish.

In the pre-cooked minces, progressive decrease of freshness is accompanied by gradual reduction of heat resistance of P.A. 3679 spores which is especially clear between 6 and 12 days of fish storage. Outgrowth of thermally injured spores in test cans was observed after different periods of incubation at 37°C. In this connection, the frequency of can swelling was compared in four groups of incubation times e.g. when 1-5, 6-10, 11-100 and 101-700 days of incubation were required for the detection of spoilage. This was accomplished by determination of percentage distribution of swells among stated groups of incubation time.

For the raw fish minces comparisons were made for the two levels of heat treatment severity — Fig. 3 for $F_0$ values not exceeding 2.0 min., and Fig. 4 for more severe processes of $F_0$ = 2.0-4.0. They were made in the function of storage time, separately for $10^6$ spores per can (Fig. 3A and 4A), and separately for $10^2$-$10^5$ spores per can (Fig. 3B and 4B), and also for all 5 concentrations together (Fig. 3C and 4C).

When milder processes are compared (Fig. 3) gradual extinction of ability for rapid 1-5 days swelling of test cans could be seen. With extension of storage time there is a tendency of gradual increase in number of cans showing swells after 6-10 days of incubation. Freshness had rather minor effects upon changes of fraction swelling after 10-100 days of incubation. On the other hand, the lack of swells during 101-700 days of incubation is characteristic for storage longer than 3 days.

For more severe heat treatment (Fig. 4) the extension of storage time is accompanied by rapid extinction of swells detected during 1-5 days of incubation, followed by increase of number of swells detected after 6-10 and 11-100 days. All but one period of storage had shown limited percent of swells in the group 101-700 days of the incubation time.

Similar comparison of distribution of swells was prepared for the pre-cooked minces (Fig. 5). For these results no distinction was made

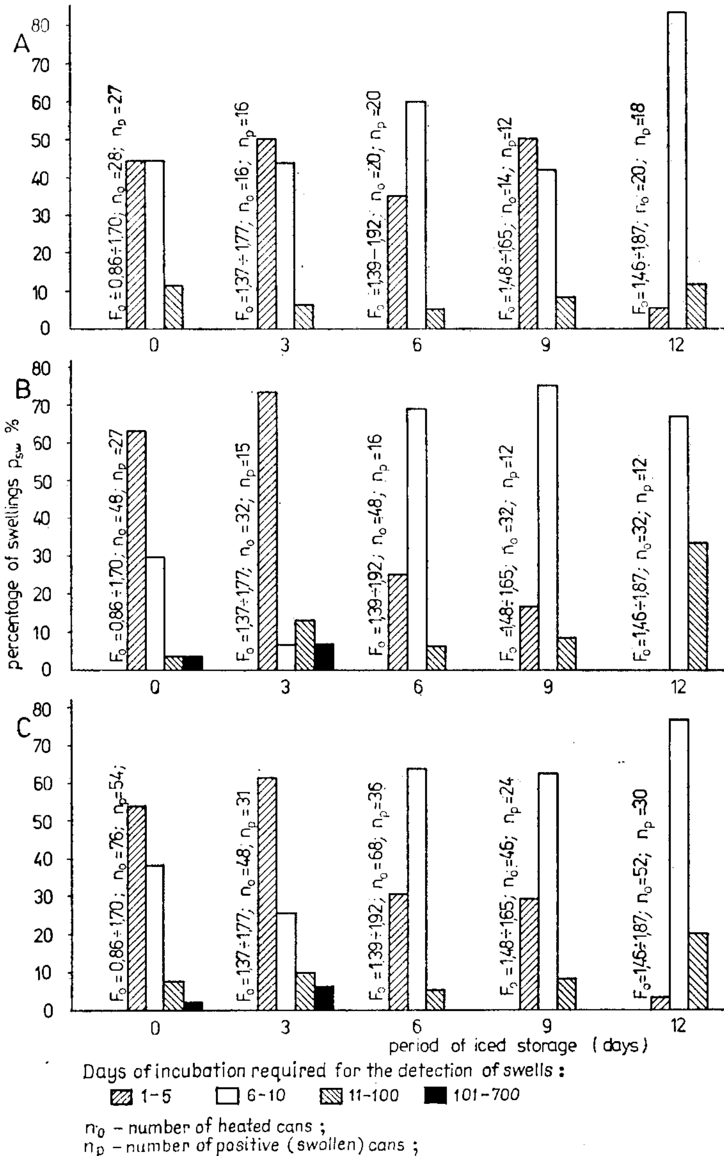


**Fig. 3.** Outgrowth of P.A. 3679 spores subjected to heat treatment of $F_0 \leqslant 2.0$ min., in the raw cod minces of different freshness; A — $10^6$ spores per container, B — $10^2$-$10^5$ spores per container, C — totally for $10^6$ and $10^2$-$10^5$ spores per container

between process severity and spore initial number per container, so presented comparison enfolds process lethal values of $F_0$ = 1.0-4.0 min and numbers of $10^4$, $10^5$ and $10^6$ spores per container. It was a result of much higher effectiveness of heat treatment of the precooked minces which was accompanied by lower number of detected swells. For this reason, the use of similar pattern of comparison as made for the raw minces was unadvisable, because that would result in an excessive dispersion of results, making partial comparison non-representative.

It is characteristic for data presented on Fig. 5 that independently from period of storage, percentages of swells detected after 6-10 and 11-100 days have approximate values, and that prevailing number of swells

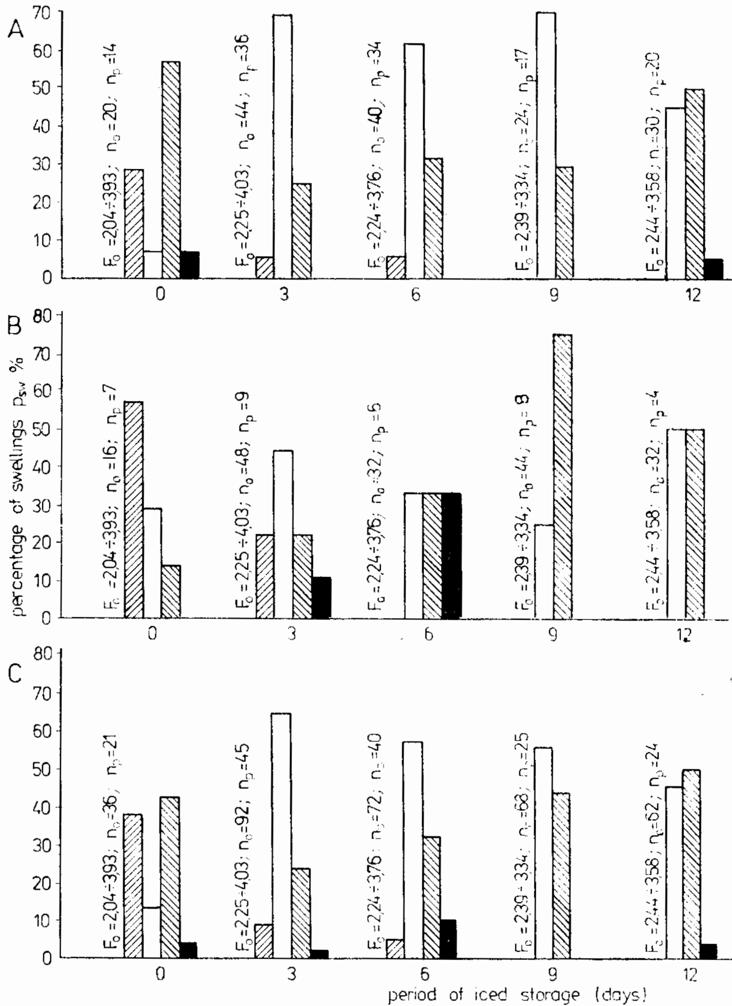


Fig. 4. Outgrowth of P.A. 3679 spores subjected to heat treatment of $F_0$ = 2.0-4.0 min., in the raw cod minces of different freshness (explanations so as Fig. 3)

falls into those two groups. Percentages of swells belonging to two remaining groups of incubation time are approximate, but on the other hand, lack of swells detected during 1-5 days for two periods of storage, e.g. 6 and 9 days could be noted.

It should be also mentioned, that none of the control samples not containing P.A. 3679 was spoiled during 700 days incubation. This may be interpreted as a low natural contamination of stored fish by heat resistant spores of mesophilic putrefractive anaerobes.

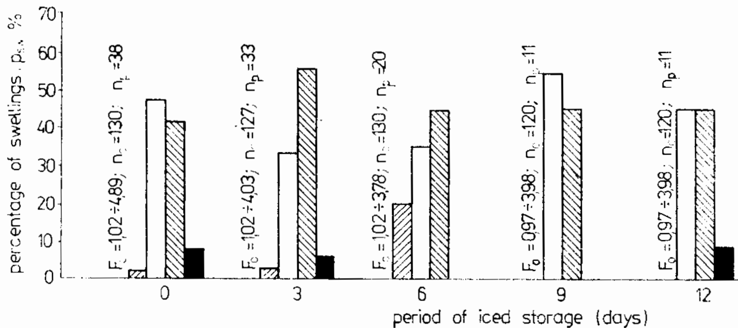


Fig. 5. Outgrowth of P.A. 3679 spores *) to heat treatment of $F_0$ = 1.0-4.0 min., in the pre-cooked cod minces of different freshness (explanations so as Fig. 3)
*) number of spores $10^4$-$10^6$ per container

## DISCUSSION

In production technology of canned foods having high water activity, the destruction of bacterial spores by moist heat is considered as the one of more efficient methods of their inactivation. Efforts aimed to increase efficiency of this method, without raising its severity but rather with use of milder processes are presently considered as difficult taks because of biological properties of spores [8, 10].

Technique applied in this study fulfills number of conditions set by present status of knowledge [6].

The use of exponentially increasing P.A. 3679 spore loads in paralelly heated samples, made possible clear determination of spore concentration influence upon effectiveness of heat treatment. It was achieved by observation of maximal heat resistance of numerically different spore populations in the tested environments.

Results of heat resistance determinations of P.A. 3679 spores suspended in the raw and pre-cooked cod minces confirmed dependences observed earlier by Alderman et al. [1] with respect to heat inactivation of *Cl. botulinum* type E spores in fish. It could be concluded from our study that when fresh fish is used as raw material in canning, the higher

heat resistance of spores should be expected when fish enters the final heat treatment in a raw state. However, on the basis of heat resistance determinations which were made in raw and pre-cooked cod mince, it could be supposed that when preliminary heat treatment is applied to fish before canning, spore load carried by raw fish could have more pronounced effect upon shelf stability of product, than similar contamination of pre-cooked fish on the processing line.

It seems that with gradual decrease of freshness, the applied standard heat denaturation of cod minces had progressively eliminated physical protective action of this substrate, exerted on the heated spores. This is proved by gradual decrease of heat resistance of P.A. 3679 spores in the pre-cooked cod minces representing increasing periods of storage. The observed patterns of outgrowth of spores, following heat processing, show dependence between number of spores initially present in product, severity of heat treatment, and period of fish storage. Most of results does not fit known rules of log normal distribution of times required for spoilage detection [10], only in a few cases of heating in milder range of lethal values, results resemble this type of distribution.

Effect of fish freshness upon heat resistance of the test organism, was rather weakly expressed if its estimation was based on the quantitative spoilage data. But on the other hand, differential dynamics of spore outgrowth in spoiling cans, expressed as an uneven distribution of times required for spoilage detection, makes visible effect of freshness upon heated spores.

Use of the raw material of superior quality is one of the basic requirements of the present fish canning technology. Also preliminary heat treatment of fish is an operation widely used in modern technology, mostly for preliminary dehydration of meat tissue. It was shown in this study that high quality of raw material, and preliminary heat treatment of fish, could be considered as factors increasing sporicidal action of heat processing.

## CONCLUSIONS

1. Number of spores introduced to cod mince is a factor determining effectiveness of heat treatment. In the raw minces 90% reduction of initial spore load leads to 30% increase of percentage of stable units, independently from severity of heat treatment.

2. Heat resistance of P.A. 3679 spores heated in initially denaturated cod mince is much lower than that shown in the raw minces.

3. Freshness of cod has rather minor effect upon heat resistance of spores heated in raw minces. Its effect is much more distinctive in the initially denaturated minces.

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## WPŁYW ŚWIEŻOŚCI RYB BAŁTYCKICH NA CIEPŁOOPORNOŚĆ SPOR BAKTERYJNYCH

### II WPŁYW ŚWIEŻOŚCI LODOWANEGO DORSZA NA CIEPŁOOPORNOŚĆ SPOR BEZTLENOWCA GNILNEGO P. A. 3679 ZAWIESZONYCH W SUROWYM I ZDENATUROWANYM CIEPLE FARSZU.

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### Streszczenie

Dorsze bałtyckie pochodzące z łowisk w rejonie Dziwnowa przechowywano w lodzie w temperaturze 0-4°C, do 12 dni od momentu złowienia. Sukcesywnie pogarszającą się świeżość reprezentowały farsze z ryb sporządzane w 3-dniowych odstępach czasu. Farsze te w stanie surowym oraz wstępnie zdenaturowanym cieplnie stanowiły środowiska, w których określano oporność termiczną spor P.A. 3679. Oznaczano ją techniką puszkową stosując procesy termiczne o wzrastającej letalności. Obróbka termiczna opakowań testowych odbywała się w warunkach symulujących sterylizację przemysłową w 110°C. W tych samych procesach równolegle ogrzewano wzrastające wykładniczo populacje spor (5 poziomów liczebności/opakowanie). Testy przeżycia prowadzono metodą inkubacji w produkcie przez 700 dni w temperaturze 37°C. No-

towano ilości bombaży i czas inkubacji, w których się one ujawniały. Badania wykazały, że pogarszająca się świeżość surowca obniża ciepłooporność spor w farszu wstępnie zdenaturowanym termicznie (spadek $D_{110.1}$ od 4.33 min wyjściowo do 2,41 min. po 12 dniach). W farszu surowym, gdzie spory wykazywały znacznie wyższą oporność termiczną ($D_{110.1}$ = 5.74-6.62 min) zależność ta była słabo wyrażona. Czasy inkubacji wymagane w celu ujawnienia bombaży były uzależnione od ilości spor/opakowanie, świeżości surowca i intensywności obróbki termicznej. Procent prób trwałych wykazał stałą zależność od $\log_{10}$ inicjalnej liczby spor/opakowanie na różnych poziomach intensywności obróbki termicznej. Badania wykazały, że wymagania stawiane współcześnie surowcowi rybnemu do produkcji konserw oraz stosowana technologia wspomagają efektywność procesów termicznych rybnych produktów konserwowych.