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Survival of Bacterial Strains in Fish Feeds Stored at Different Temperatures

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Abstract

Studies were carried out on the survival of six strains of bacteria: *Pseudomonas fluorescens, Pseudomonas aeruginosa, Escherichia coli, Streptococcus faecalis, Bacillus subtilis* and *Bacillus cereus* in fish feeds stored for 72 days at -11°C, 5°C and 20°C. On the last day of the study live cells of all bacterial strains were found in the feed stored at -11°C, three strains at 5°C, and two at 20°C. Two strains, *Bacillus subtilis* and *Bacillus cereus* survived throughout the entire storage period at all three temperatures.

Keywords: *Pseudomonas fluorescens, Pseudomonas aeruginosa, Escherichia coli. Streptococcus faecalis, Bacillus subtilis, Bacillus cereus,* survival, fish feed

Introduction

Composition of the microflora in fish food tract depends most of all on the environment and the diet. In fish culture artificial feeds are used along with natural food [1, 11]. Microorganisms present in the fish feed may cause its decomposition or cause fish diseases. Hence, microbiological quality of the feeds is of considerable importance.

Microorganisms present in the feeds may be characterized by different survival depending on the chemical composition of the feed and storage conditions. Consequently, feeds used in aquaculture should be controlled for their microbiological quality according to the existing standards. Currently Polish Standard PN-76/R-64791 is used [15]; it refers to dry feeds in which proteolytic and ammonifying bacteria must be determined as well as saprophytic and toxin-producing fungi.

No papers have been found in available literature on the survival of microorganisms in fish feeds stored at different temperatures. Hence, it seemed appropriate to carry out studies on these problems, with special attention given to the microorganisms mentioned in the above standards.

The objective of the study was to determine the survival of six strains of bacteria: *Pseudomonas fluorescence, Pseudomonas aeruginosa, Escherichia coli, Streptococcus faecalis, Bacillus subtilis, Bacillus cereus* in fish feed stored at three temperatures: -11°C, 5°C and 20°C. With the exception of *Streptococcus faecalis,* all strains are characterized by proteolytic properties, which cause deterioration of products containing proteins.

Materials and Methods

The materials consisted of:

1. Fish feed containing: yeast, wheat flour, fish meal, meat-bone meal, fish oil, minerals (Polfamix B, Polfa-Kutno) and vitamin mixture **[14].**

2. **Bacterial strains:** *Pseudomonas fluorescence* T 88, *Pseudomonas aeruginosa* W2, *Escherichia coli* T205, *Stre ptococcus faecalis* T17, *Bacillus subtilis* T95 and *Bacillus cereus* T93 (strains originated from strain collection of the Department of Commercial Microbiology and Food of the University of Agriculture and Technology in Olsztyn). All strains were lyophilized.

Methods consisted of the following stages:

1. Preparation of bacterial suspensions. Lyophilized strains of bacteria were grown on nutritive broth for 2 hours. Then 1 ml of each bacterial suspension was added to 50 ml of broth and incubated for 24 hours at a temperature optimal for a given strain. The obtained broth bacterial cultures were centrifuged for 10 min. at 10 rot./min. in a Janetzki centrifuge T24. Liquid above the sediment was removed with a pipette, the procedure being repeated twice using physiologic saline.

2. **Sample preparation.** Six 30 g weighted portions of sterile fish feed were prepared. Sediment obtained after culture centrifugation was added to each portion and thor oughly mixed. The obtained inoculated feed samples were divided into weighted 1 g portions and placed in 30 test-tubes for each strain of bacteria. Ten samples were placed at each of the three temperatures: -11°C, 5°C and 20°C.

3. **Microbiological analyses of the feed samples.** The first quantitative analysis was performed immediately after sample inoculation with bacteria, lg portions were analy zed. Subsequent analyses were carried out at 8-day inter vals until day 72. Each time 1 g portions were taken from each storage temperature. Quantitative determinations were made using the plate method and selective media for par ticular bacterial strains (Tab. 1). 0.85% NaCl solution was used as the diluent.

All analyses were made in three repetitions. Colonies were counted after incubation determining CFU per 1 g of the feed. Survival of bacteria was determined in days and percentages.

Results

Figs. 1 to 6 present curves illustrating survival of the six bacterial strains in fish feed stored for 72 days under three different temperatures. Numbers of bacteria are presented using a logarithmic scale.

Pseudomonas fluorescens (Fig. 1) and *Escherichia coli* (Fig. 3) were characterized by the shortest survival in 5°C and 20°C. All bacterial cells died before the end of the experiment. No live cells of *Pseudomonas fluorescens* were observed after 40 days and of *Escherichia coli* after 56 days at 5°C. At 20°C there were no live cells of these two strains already after 24 days. The temperature of -11°C was better tolerated by these bacteria; live cells were found even on the last day of the experiment: 750 CFU per 1 g of the feed in the case *of Pseudomonas fluorescens* and 1800 in the case of *Escherichia coli.* The two strains showed slow but regular mortality throughout the experiment.

Mortality of *Pseudomonas aeruginosa* (Fig. 2) was more rapid at the beginning of the experiment, until day 32. Later, until day 64, numbers of these bacteria were more or less uniform under all storage temperatures. On the last day of the experiment there were no live cells found at 5°C and 20°C, while at -lT'C there were still 120 CFU per 1 g of the feed.

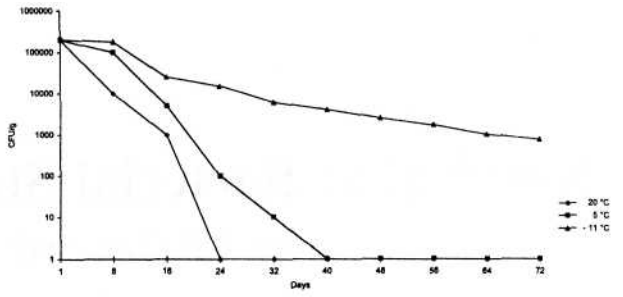

Fig. 1. Survival of *Pseudomonas fluorescens* in fish feed.

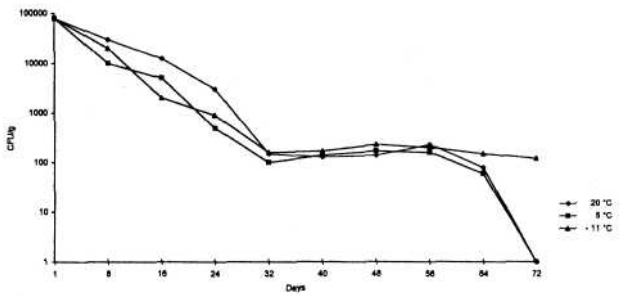

Fig. 2. Survival of *Pseudomonas aeruginosa* in fish feed.

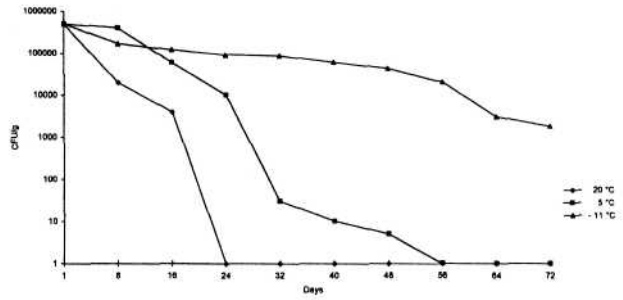

Fig. 3. Survival of *Escherichia coli* in fish feed.

Survival of *Streptococcus faecalis* (Fig. 4) in the feed stored at 20°C differed considerably from that in the other two storage temperatures. Mortalities were higher at 20°C. On day 64 no live cells were observed in the feed. Feed stored at lower temperatures still contained live cells of *Streptococcus faecalis* on the last day: 2500 per 1 g of the feed at 5°C and 29800 at -11°C.

Table 1.

Bacterial strain	Incubation	Medium	Source
Pseudomonas fluorescens	25°C/72 h	Kinga B	Burbianka, Pliszka [2]
Pseudomonas aeruginosa	42°C/48 h	Pseudomonas Agar F	Medium produced by "Difco"
Escherichia coli	37°C/48 h	Endo	Burbianka, Pliszka [2]
Streptococcus faecalis	37°C/48 h	HF Streptococcus Agar	Medium produced by "Difco"
Bacillus subtilis	25°C/48 h	Broth agar with glucose	Burbianka, Pliszka [2]
Bacillus cereus	25°C/48 h	Broth agar with glucose	Burbianka, Pliszka [2]

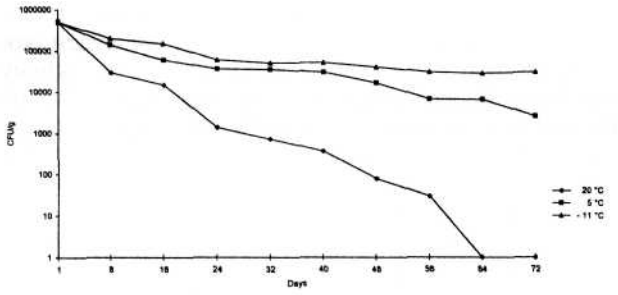

Fig. 4. Survival of *Streptococcus faecalis* in fish feed.

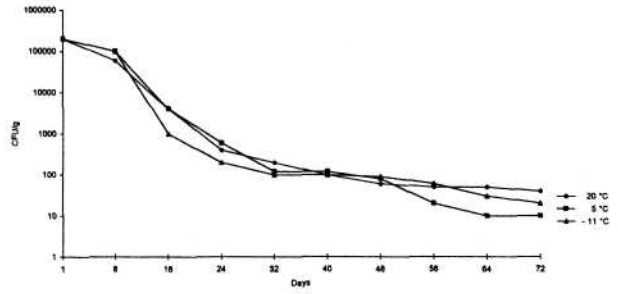

Fig. 5. Survival of *Bacillus subtilis* in fish feed.

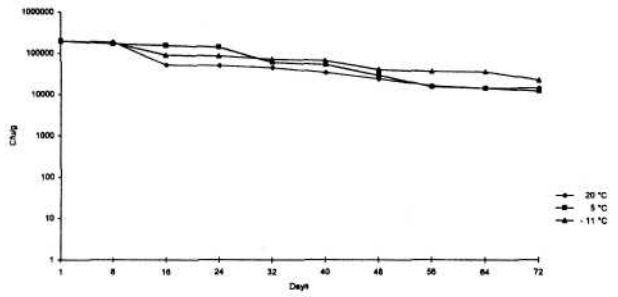

Fig. 6. Survival of *Bacillus cereus* in fish feed.

Survival of *Bacillus subtilis* (Fig. 5) was similar under all storage temperatures. Higher mortalities were observed until day 24; later numbers of bacteria decreased very slowly. On the last day of the experiment there were a few tens of CFU per 1 g of the feed.

The highest survival was observed for *Bacillus cereus* (Fig. 6). Numbers of these bacteria decreased very slowly and at a similar rate under all storage temperatures. On the last day there were still 21800 live cells in 1 g of the feed stored at -11°C, 12000 in that stored at 5°C, and 14500 at 20°C.

Table 2 presents the survival of the six bacterial strains in days and percentages, and at the three temperatures of storage.

All strains survived the entire experiment (72 days) at -11°C. The highest percentage of survival (10.9%) was found for *Bac. cereus;* the lowest (0.01%) for *Bac. subtilis.* Other percentages were 6.00, 0.38, 0.36 and 0.15, respectively, for *Str. faecalis, P. fluorescens, E. coli* and *P. aeruginosa.*

At 5°C half of the strains survived 72 days of storage. The highest percentage survival of 6.00% was observed for *Bac. cereus,* 0.5% for *Str. faecalis,* and 0.005% for *Bac. subtilis.* No live cells of *P. aeruginosa* were found after 72 days, of *E. coli* after 56 days, and of *P. fluorescens* after 40 days of feed storage at this temperature.

At 20°C survival of *Bac. cereus* amounted to 7.25% after 72 days of feed storage, of *Bac. subtilis* to 0.02%. *P. aeruginosa* showed 0 % survival after 72 days, *Str. faecalis* - after 64 days, and *P. fluorescens* and *E. coli* already after 24 days.

Discussion

Survival of micro-organisms is a changeable feature, depending most of all on the environment. Available literature on the survival of bacteria deals with such environments as water and foodstuffs. There are no papers devoted to microorganism survival in stored fish feeds.

The six bacteria strains studied in this experiment have also been analyzed by other authors [6, 13], but attention focused on their survival in water. Survival in foodstuffs has usually concerned only *Pseudomonas* species [7, 10].

Our studies show that *Escherichia coli* contained in fish feeds survived 56 days at 5°C, and as many as 32 days fewer at 20°C. Similar results were obtained by Me Feters et al. [12] in studies on *E. coli* survival in river water. These authors observed lower survival at 20 to 30°C than at 0 to 10°C.

Table 2. Survival of bacterial strains in fish feed (in percentages and days)

Bacteria	Initial number of bacteria per 1 g of the feed	Temperature					
		-11°C		5°C		20°C	
		%	Dni	%	Dni	%	Dni
Pseudomonas fluorescens	200,000	0.38	> 72	0	40	0	24
Pseudomonas aeruginosa	80,000	0.15	> 72	0	72	0	72
Escherichia coli	500,000	0.36	> 72	0	56	0	24
Streptococcus faecalis	500,000	6.00	> 72	0.5	> 72	0	64
Bacillus subtilis	200,000	0.01	> 72	0.005	> 72	0.02	> 72
Bacillus cereus	200,000	10.90	> 72	6.00	> 72	7.25	> 72

Similar results were obtained by Gyllenberg et al. [9] and Flint [6] who showed longer survival of *Escherichia coli* in water at 4°C than at 37°C. Hence, survival of these rods is inversely proportional to temperature. Also, Terzieva and Me Feters [16] observed longer survival of *E. coli* in spring water at 6°C than at 16°C.

Taking into account survival of particular strains, our observations revealed longer survival at 5 and 20°C of *Streptococcus faecalis* compared with *Escherichia coli.* Similar results were obtained by Me Feters et al. [12]. These authors found that the survival of enterococci in water was higher than of *Escherichia coli.* Zmyslowska [17] stated that survival in lake water containing peptone was 2 days longer for *Escherichia coli* and 4 days longer for *Streptococcus faecalis* at 4°C compared to 20°C. At higher temperatures reduction of the numbers of bacteria was more rapid for both *Escherichia coli* and *Streptococcus faecalis.* The same was observed by Faust et al. [4] and Niewolak [13] as regards survival of the same bacteria in lake water.

From among the six strains of bacteria, *Bacillus cereus* and *B. subtilis* showed the highest survival rates. Considerable resistance to temperature and environmental conditions of bacteria belonging to *Bacillus* genus are related to their ability to form spores. Fili et al. [5] obtained similar results in studies on micro-organism survival in underground water and 10°C.

Pseudomonas aeruginosa was also characterized by a much higher resistance to environmental conditions and remained alive throughout the whole study period (72 days) at -11°C, and for 64 days in the other two temperatures. Filip et al. [5] stated that *Pseudomonas aeruginosa* survived in underground water for 100 days at 10°C.

Our studies showed that *Pseudomonas fluorescens* cells remained alive in fish feed for the whole period of 72 days only at -11°C. At 5° and 20°C these bacteria disappeared after 40 and 24 days, respectively. Gennari et al. [8] obtained similar results for *Pseudomonas fluorescens* at low temperatures, in sardines stored in a refrigerator. Gennari and Dragotto [7] also observed survival of *Pseudomonas fluorescens* in meat, fish, milk, cheese, soil and water.

It can be concluded that all strains of bacteria *(P. fluorescens, P. aeruginosa, E. coli, Str. faecalis, Bac. subtilis, Bac. cereus)* were characterized by the highest survival at -11°C. Feed samples stored at this temperature contained live bacterial cells even on the last day of the experiment. Especially high survival was observed for two spore-producing strains: *Bac. subtilis* and *Bac. cereus.* Live cells of these strains were found on the last day in feed samples stored at all temperatures (-11, 5 and 20°C).

With the exception of *Streptococcus faecalis,* all bacteria introduced into feed samples showed proteolytic abilities. Proteolytic microflora is characterized by a definite activity to decompose proteins. High temperature reduces microorganism survival, but enhances proteolytic activity (Donderski [3] cit. after Sandvik and Fossum, Christison and Martin, Ambroz). This fact is of considerable importance in food storage. Islam and Blaushard (cit. after Donderski [3]) stated that bacteria from *Bacillus* genus showed maximal proteolytic activity at 40°C. Juffs [10] found that proteases produced by *Pseudomonas aeruginosa* showed the highest proteolytic activity at 30°C, while *Pseudomonas fluorescens* - at 20°C. The results obtained by Donderski [3] revealed that bacteria belonging to the genera *Pseudomonas* and *Bacillus* were characterized by the highest proteolytic activity at 37°C, while at 20 and 26°C this activity was lower. Another effect of higher temperature (37°C) on the activity of proteases produced by bacteria isolated from lake water was observed for the species belonging to the family *Enterobacteriaceae.*

In view of the above it may be concluded that microbiological examination of fish feed quality should take into account the presence of proteolytic bacteria, especially spore-producing ones, as these are likely to affect the quality of feed proteins. Attention should also be given to storage temperature, which affects survival of bacteria and their enzymatic activity.

Conclusions

1. All strains of bacteria examined in this study *{Pseudomonas fluorescens, Pseudomonas aeruginosa, Escherichia coli, Streptococcus faecalis, Bacillus subtilis, Bacillus cereus)* showed the highest percentage and the longest survival in fish feed stored at -11°C, in which live bacterial cells were observed till the end of the experiment (for 72 days).

2. Microbiological examination revealed that fish feed should be stored at 5° or 20°C. In these temperatures bacteria showed lower survival in (both percentages and days) than at -11°C.

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