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MICROFLORA OF LOW-SALT HERRING II. THE INFLUENCE OF SODIUM BENZOATE ON MICROFLORA OF LOW-SALT HERRING

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ABSTRACT

Results of qualitative and quantitative studies on microflora of low-salt herring slices supplemented with 0%, 0.2% and 0.3% of sodium benzoate (E-211) are presented. Herring slices were subjected to low salting with addition of 0%, 0.2% and 0.3% of sodium benzoate according to a recipe provided by a local manufacturer. It was observed that sodium benzoate reduced diversity of bacteria and yeasts in a tested product and exerted no influence on the total number of bacteria and yeasts. It gave the evidence that an empty ecological niche was created after elimination of some species by the preservative and remainders substituted them. As a result no reduction of the total number of micro-organisms was observed and a shelf life of the product was not prolonged. Our results proved that sodium benzoate is the ineffective preservative for low-salt herring production.

Key words: bacteria, yeasts, salt herring, sodium benzoate (E-211)

INTRODUCTION

Sodium chloride (NaCl) is one of the oldest substances known to be used by human to improve taste and protect food against spoiling. Two basic methods of salting: low and high are generally applied. Products subjected to high salting are protected against deterioration by micro-organisms and may be stored over 10 months at 10-12°C. If temperature of storage is lowered from 5°C to 0°C, their shelf life may last even over a year [15]. Changes within microflora take place during high salting. Halophilic bacteria begin to dominate as long as they

reach a stable level. However, they do not participate in product spoiling. A traditional way of high salting is believed to eliminate most of human pathogens as no cases of food-borne infections connected with consumption of high-salt herring have been reported.

Presently, low salting (10-15% of NaCl in saline with/without addition of sugar and spices) is more often applied. The concentration of salt in fish tissue salted that way fluctuates from 5% to 7%. Products protected by this method cannot be considered as safe for consumers because they may create convenient conditions for pathogens to grow [3, 5]. It is suggested that bacteria present in such products were causative agents of many food poisonings [1]. In contrary to high-salt, low-salt herring has a short shelf life and gradually gets spoiled during storage because of constantly increasing number of micro-organisms. It is the reason to apply additional substances supporting a protective activity of sodium chloride.

Benzoic acid (E-210) is one of the earliest synthetic preservatives used. Due to its low solubility it is applied as sodium benzoate [10]. Benzoic acid and its salts (E-210-219) present a good inhibition effect on yeasts and moulds, worse on butyric bacteria and hardly any effect on lactobacilli. Their antimicrobial activity is expressed at low range of pH (optimum 2.5-4.0) [4, 9, 11]. Some authors suggest it may be extended up to 4.5 [13].

Application of chemical preservatives should be only supporting and at reasonably justified concentrations. Changes of microflora in high-salt herring are relatively well studied. Unfortunately, after introducing low-salt herring into the market, no sufficiently thorough and close microbiological studies of the product were conducted. The influence of added preservatives on protection of low-salt herring was not also examined. Due to it, the aim of our studies was to test the effect of various concentration of sodium benzoate (E-211) on microflora of low-salt herring.

MATERIALS AND METHODS

Slices of herring (*Clupea harengus harengus* L.) frozen in blocks were used. They were salted according to a recipe provided by a local producer. Brine (pH 4.75) consisted of salt (8.4 kg), 10% vinegar (4 L), salinate – an enzymatic preparation (0.3 kg) and water (60 L). In our studies it was also supplemented with sodium benzoate to obtain concentrations of 0%, 0.2%, 0.3%, respectively.

Herring was salted in brine (1:1) in plastic boxes (10 pieces each) and stored at 8°C and 20°C. Microbiological analyses of fish and brine were performed twice a week. Composition of the total microflora and pH were tested.

According to Polish Standard (PN-89 A-86730) 180 ml of a dilution liquid (peptone tryptone /Difco/ 1.0 g, sodium chloride 8.5 g, distilled water 1000 ml, pH 7.2-7.4) was added to 20-g samples of non skinned fish tissues collected in a sterile way. Samples were homogenised in a stomacher for 3 minutes and their serial decimal dilutions were prepared. Then, 0.1 ml was inoculated on appropriate medium for quantitative analyses. The total number of microorganisms grown on nutrient agar (BTL, Poland) after incubation at 30°C was counted. Preliminary identification of colonies was based on Gram staining.

Genus and species identification for Gram-negative rods was performed on ID 32 GN test, for Gram-positive rods on API Coryne test and for Gram-positive cocci on ID 32 STAPH test (bioMèrieux).

The number of yeasts in tested samples was also analysed by inoculation of serial dilutions directly on agar medium for quantitative analyses of yeasts and moulds (yeast extract 5.0 g, glucose 20.0 g, chloramphenicol 0.1 g, agar 15 g, distilled water 1000 ml, pH 6.6) according to PrPN-ISO 7954. Inoculated plates were incubated for 3-5 days at ambient temperature. Then, their genus and species identification was carried out using ID 32C test (bioMèrieux).

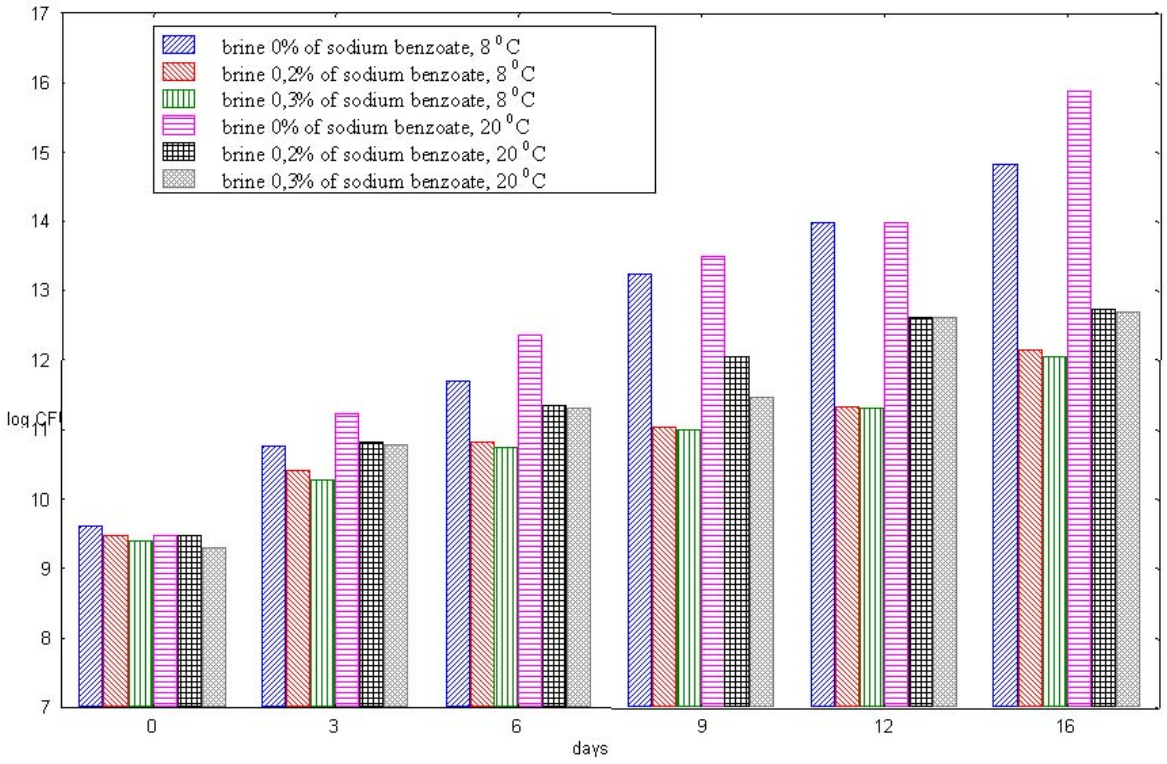
Biochemical results were read and compared in VITEK System ATB Expression (bioMèrieux).

Statistic analyses, i.e. mean, standard deviation, Scheffe test, were performed with Statistica PL software at a confidence limit $p \leq 0.05$.

RESULTS

Analyses of the total number of bacteria in herring samples salted without sodium benzoate and stored at 8°C and 20°C showed a gradual increase in their quantity. Statistically significant differences were observed on 12th day of incubation. Similarly, slices salted with addition of 0.2% of sodium benzoate and stored in the same conditions presented a significant increase in the number of mesophilic microorganisms on 12th day of analyses. Statistically significant differences were also found in the number of mesophiles among samples with or without sodium benzoate. Results of analyses of the total number of microorganisms are presented in Figure 1.

Figure 1. Changes in the total number of bacteria in fish depending



Subsequent analyses were carried out to identify isolated strains at the genus and species level. In salt fish without and with 0.2% of sodium benzoate seven genera of bacteria were recognised, in contrary to fish salted with 0.3% of sodium benzoate where only two genera were present. *Acinetobacter* sp. (35% of isolates) and *Lactococcus* sp. (30% of isolates) dominated in fish salted without the preservative and stored at 8°C. *Streptococcus* sp. and *Proteus* sp. were mainly found in herring stored at 20°C and consisted of 35,7% and 21.4% isolated strains, respectively (Table 1).

In samples salted with 0.2% of sodium benzoate and kept at 8°C *Acinetobacter* sp. (32.4% of isolates) and *Lactococcus* sp. (32.4% of isolates) as well as *Escherichia* (26.5% of isolates) were mainly cultured. Incubation at 20°C promoted growth of *Lactococcus* sp. and *Streptococcus* sp. (25% of isolates, respectively) (Table 2).

Table 1. Genera of bacteria isolated from salt fish without sodium benzoate added

Genus	Incubation temperature			
	20°C		8°C	
	10^4CFU/g	%	10^5CFU/g	%
Acinetobacter	3.0	7.1	7.0	35.0
Aeromonas	6.0	14.3	0.0	0.0
Escherichia	3.0	7.1	4.0	20.0
Pseudomonas	0.0	0.0	2.0	10.0
Proteus	9.0	21.4	0.0	0.0
Lactococcus	6.0	14.3	6.0	30.0
Streptococcus	15.0	35.7	1.0	5.0

Table 2. Genera of bacteria isolated from salt fish with 0.2% sodium benzoate added

Genus	Incubation temperature			
	20°C		8°C	
	10^4 CFU/g	%	10^5 CFU/g	%
Acinetobacter	3.0	15.0	11.0	32.4
Aeromonas	4.0	20.0	0.0	0.0
Escherichia	0.0	0.0	9.0	26.5
Pseudomonas	0.0	0.0	2.0	5.9
Proteus	3.0	15.0	0.0	0.0
Lactococcus	5.0	25.0	11.0	32.4
Streptococcus	5.0	25.0	1.0	2.9

Fish samples salted with 0.3% of sodium benzoate and stored at 8°C created excellent medium for growth of *Acinetobacter* sp. (96.7% of isolates). *Pseudomonas* sp. (54.1% of isolates) and *Acinetobacter* sp. (45.9% of isolates) were predominantly found in samples stored at 20°C (Table 3).

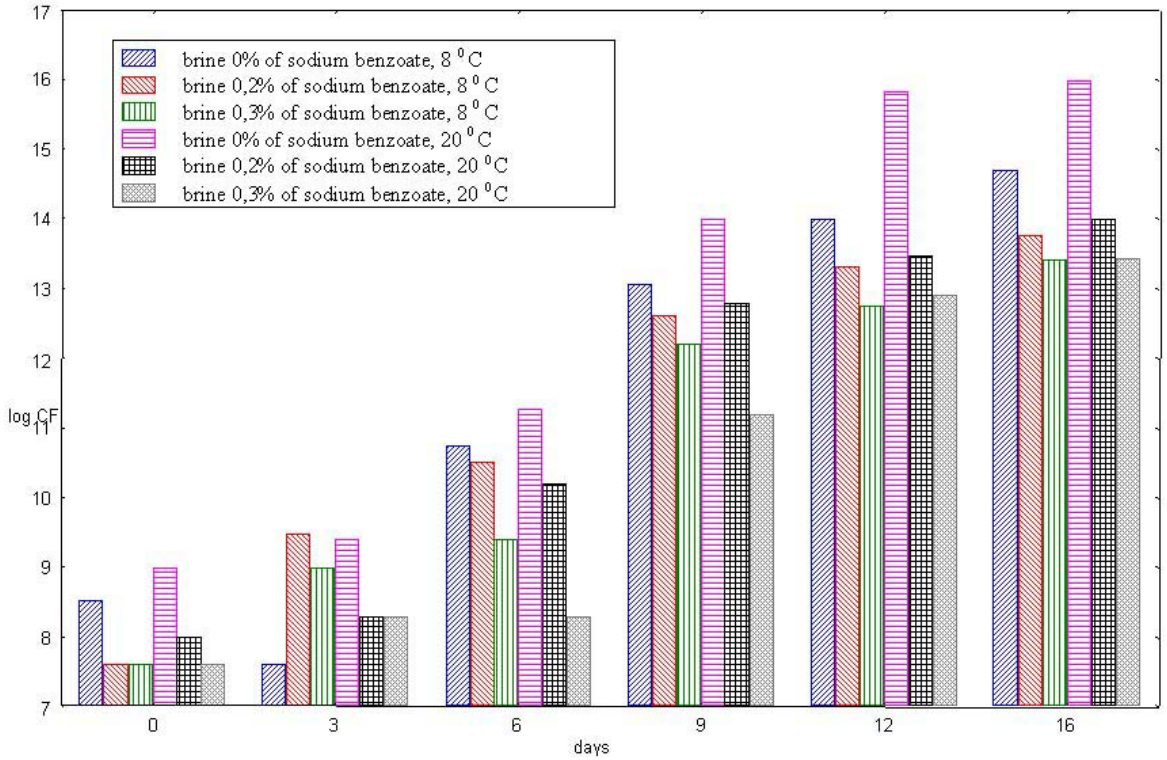
Quantitative analyses of yeasts showed significant differences on 16th day of examination in samples without sodium benzoate stored at 8°C and on 12th day in samples kept at 20°C.

Table 3. Genera of bacteria isolated from salt fish with 0.3% sodium benzoate added

Genus	Incubation temperature			
	20°C		8°C	
	10^3 CFU/g	%	10^4CFU/g	%
Acinetobacter	78.0	45.9	29.0	96.7
Pseudomonas	92.0	54.1	1.0	3.3

The significant increase in the number of yeasts occurred already on 9th day of examination in slices salted with 0.2% of sodium benzoate and stored at 8°C. At 20°C rise in the number of yeasts was not seen before 12^{th} day of analyses. A significant increase was observed on 12^{th} day of storage at 8°C and 20°C in samples with 0.3% of sodium benzoate. Statistically significant differences were observed among samples with or without addition of the preservative. No differences were found between the number of yeasts in herring at 8°C and 20°C. Results of analyses are presented in Figure 2.

Figure 2. Quantitative changes in fish yeasts depending on concentration of sodium benzoate and incubation temperature



Herring salted without the preservative added and kept at 8°C promoted growth of *Candida* sp. (67.5% of isolates). *Candida* sp. (46.7% of isolates) and *Pichia* (20% of isolates) dominated in samples stored at 20°C (Table 4).

Table 4. Genera of yeasts isolated from fish without sodium benzoate added

Genus	Incubation temperature			
	20°C		8°C	
	10^5 CFU/g	%	10^4 CFU/g	%
Candida	35.0	46.7	81.0	67.5
Cryptococcus	6.0	8.0	0.0	0.0
Pichia	15.0	20.0	0.0	0.0
Rhodotorula	6.0	8.0	0.0	0.0
Sporobolomyces	13.0	17.3	19.0	15.8
Trichosporum	0.0	0.0	20.0	16.7

Similar results were observed in fish samples salted with 0.2% of sodium benzoate and kept at 8°C. *Candida* sp. composed 66.7% of the total microflora. Both *Candida* sp. (42.8% of isolates) and *Pichia* sp. (21.4% of isolates) dominated at 20°C (Table 5).

Table 5. Genera of yeasts isolated from fish with 0.2% sodium benzoate added

Genus	Incubation temperature			
	20°C		8°C	
	10^4 CFU/g	%	10^4 CFU/g	%
Candida	30.0	42.8	40.0	66.7
Cryptococcus	6.0	8.6	0.0	0.0
Pichia	15.0	21.4	0.0	0.0
Rhodotorula	6.0	8.6	0.0	0.0
Sporobolomyces	13.0	18.6	10.0	16.7
Trichosporum	0.0	0.0	10.0	16.7

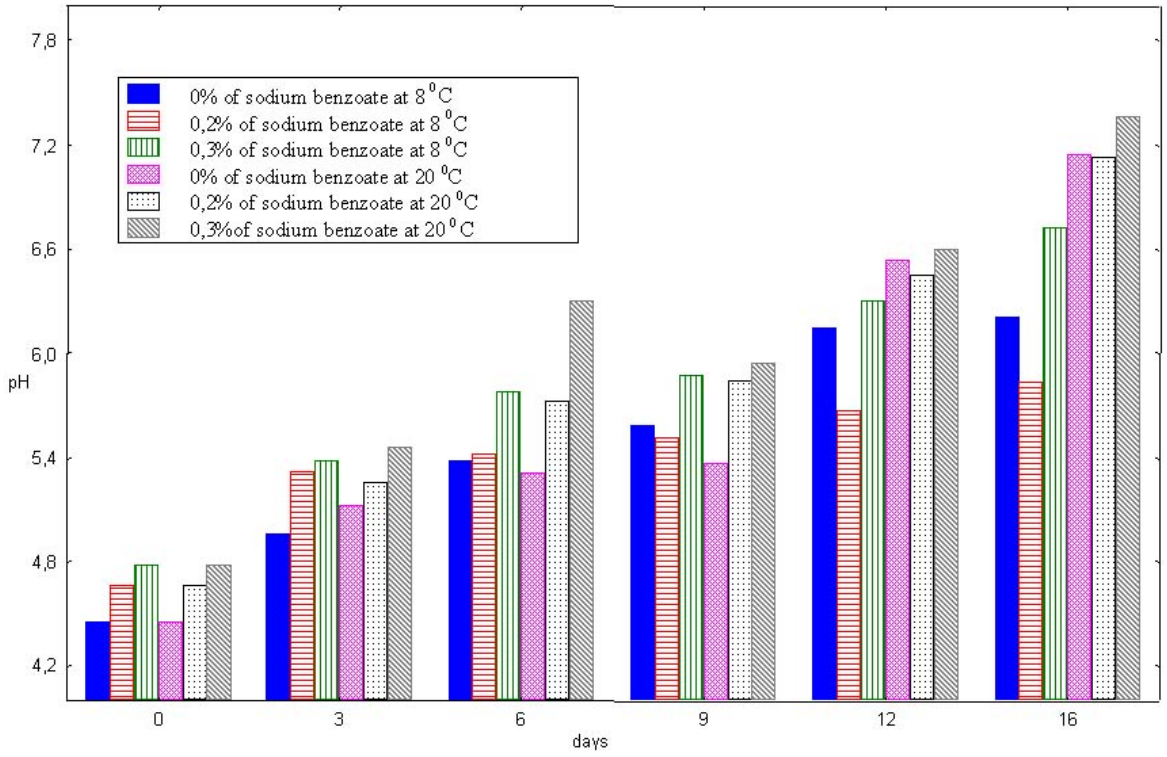
Only two genera of yeasts: *Candida* (58.8% of isolates) and *Sporobolomyces* (41.2% of isolates) were isolated from herring salted with 0.3% of the preservative and stored at 8°C. Five genera were found in samples kept at 20°C with the same genera dominating, i.e. *Candida* (33% of isolates) and *Sporobolomyces* (31.3% of isolates) (Table 6).

Table 6. Genera of yeasts isolated from fish with 0.3% sodium benzoate added

Genus	Incubation temperature			
	20°C		8°C	
	10^4 CFU/g	%	10^4 CFU/g	%
Candida	22.0	33.0	20.0	58.8
Cryptococcus	8.0	11.9	0.0	0.0
Pichia	0.0	0.0	0.0	0.0
Rhodotorula	7.0	10.4	0.0	0.0
Sporobolomyces	21.0	31.3	14.0	41.2
Trichosporum	9.0	13.4	0.0	0.0

Changes of pH during sample storage were also checked in particular conditions. In every case the slower pH increase at 8°C than at 20°C was observed. The highest pH at 8°C was observed in samples supplemented with 0.3% of sodium benzoate, the lowest at 0.2% of the preservative. The highest pH increase was observed in case of samples without the preservative tested, the lowest in samples with 0.2% of sodium benzoate, both stored at 20°C. However, pH changes were not statistically significant (Fig. 3).

Figure 3. pH changes depending on changes in sodium benzoete concentration and storage temperature



DISCUSSION

Almost 40 years ago Shewan [14] stated that the number of bacteria initially increased during high salting at 15°C. After 15 days a systematic decrease was observed, mainly among halophobic bacteria, then the number of bacteria, mostly halophilic cocci, stabilised and remained unchanged for a very long time. Changes in quantity of bacteria were slower in products ripened at lower temperatures (6°C-8&°C). Time needed for a population to reach stationary phase of growth was about 30 days. Organoleptic properties of high-salt fish products were also retained without necessity to add preservatives.

Presently, almost solely low salting is applied. Concentration of salt in low-salt fish is about 7%. Due to it, herring may be prepared by consumers without a need of soaking in water, as it is necessary in case of high-salt fish. However, low-salt and high-salt products differ from microbiological point of view. High-salt herring products have a long shelf life and they are microbiologically safe. Low-salt products undergo a process of slow deterioration and create convenient medium for growth of food-borne pathogens [3, 6].

The influence of sodium benzoate on microflora of low-salt herring was analysed in our studies. Samples supplemented with 0%, 0.2%, 0.3% of sodium benzoate were tested. A 0.2% concentration of sodium benzoate is the highest dose permissible in food. Application of 0.3% was used to learn if higher concentration of the preservative may prevent the product from spoiling. Samples were stored at 8°C and at 20°C, respectively. 8°C is the maximum temperature of product storage suggested by a producer (4°C-8°C) and it is also the temperature most often measured in household refrigerators. 20°C was applied because of supposition that shopkeepers and consumers may still treat low-salt herring as high-salt one, which used to be stored in barrels kept in ambient temperatures. Our results showed that salt products stored at 8°C had the shelf life of 6 weeks whereas the shelf life of samples kept at 20°C was shorter (3 weeks).

It was observed that regardless of temperature of storage in low-salt herring preserved with sodium benzoate a gradual growth of microorganisms takes place. In all three groups of products (0%, 0.2%, 0.3% of sodium benzoate) significant increase in the number of bacteria in comparison with initial values occurred on 12th day of analyses. Such results show clearly that the application of sodium benzoate does not reduce a growth rate of microorganisms and does not extend the shelf life of the product. Statistically significant differences occurred in the number of microorganisms among samples with and without the preservative added but they were microbiologically insignificant. Results of our studies give the evidence that sodium benzoate is an inefficient

preservative for low-salted herring. It may be caused by too high initial pH of fish products. Sodium benzoate expresses the best preservative activity within pH range from 2.5 to 4.0 [2, 4, 9, 11, 13]. Although brine used for salting was supplemented with 10% vinegar its initial pH was 4.3-4.7 and it was too high to create a favourable environment for the preservative to act. The negative influence of sodium benzoate on organoleptic properties of the product was also observed since a working dose is close to perceptible one [9] Even though other preservatives which do not change sensory properties are recommended for protection of fish products (e.g. lactic acid E-270, glutamic acid) [8], sodium benzoate is still widely applied by herring producers.

Our research also covered the influence of sodium benzoate on species diversity of isolated bacteria. No significant differences were found between samples without and with 0.2% of sodium benzoate. Gram-negative and Gram-positive cocci in samples without the preservative kept at 20°C comprise 50% of microflora each. The number of cocci was reduced to 35% in samples stored at 8°C. A similar phenomenon was observed after supplementing samples with 0.2% of sodium benzoate. No cocci were isolated from samples salted with 0.3% of the preservative and they were replaced by *Pseudomonas* sp. and *Acinetobacter* sp. According to Roberts [12] preservation of products with sodium benzoate causes displacing of dominant Gram-negative bacterial populations by Gram-positive microorganisms. It is intriguing that *Acinetobacter* became a dominant genus at 8°C. Elimination of other genera, e.g. S*treptococcus, Lactococcus, Proteus, Escherichia, Aeromonas* might have been provoked by higher susceptibility to the preservative. An empty niche created after elimination of particular species was colonised by strains resistant to sodium benzoate, mainly *Pseudomonas* sp. and *Acinetobacter* sp. It caused the lack of differences in the total number of bacteria.

Observed phenomena demonstrate that in samples with a little preservative added spoilage of product will be produced by putrefaction processes caused by *Proteus* sp*., Escherichia* sp., *Aeromonas* sp. and by fermentation processes caused by lactobacilli: *Lactococcus* sp. and *Streptococcus* sp. If the preservative concentration increases only putrefaction processes occurs.

A similar effect of reduction of species diversity was produced by temperature of storage. In this case low temperature caused a decrease in the total number of micro-organisms in samples.

Presence of yeasts in herring slices was also tested. No significant qualitative and quantitative differences were observed in herring salted without and with 0.2% of sodium benzoate. In both cases at 8°C three genera of yeasts were isolated: *Candida, Sporobolomyces, Trichosporum.* At 20°C *Candida, Sporobolomyces, Rhodotorula* and *Cryptococcus* were present. Only application of 0.3% of sodium benzoate eliminated *Trichosporum* at 8°C. At 20°C *Trichosporum* was replaced by *Pichia.* Kobatake [7] also observed that *Candida* and *Cryptococcus* were dominant in sea food. According to Nassar [11] growth of genus *Trichosporum* is promoted in products with sodium benzoate.

Inefficiency of sodium benzoate widely applied in preservation of low-salt herring is intriguing. The preservative is commonly added to beverages, fruit and vegetable products or mayonnaise. In such products its activity is supported by low pH (juices, drinks and fruit pulp), low water activity (mayonnaise) and other preservatives used, e.g. sorbic acid and its salts (E-200-203) or sulphur dioxide and its salts (E-220-228). It is very probable that the nutrient-rich matrix created by low-salt herring enhances repairing mechanisms of bacteria.

Results of performed analyses lead to conclusion that micro-organisms begin to multiple in low-salt herring since the first stages of their processing and products undergo constant processes of spoiling. The increase in the number of mesophiles and yeasts in tested samples appeared after 1 week and 3 weeks, respectively, before spoilage of samples was observed. It is clear, as spoilage of product must be preceded by multiplication of bacteria whereas yeasts do not play any important role in deterioration of meat and fish products. Our results showed that salt products stored at 8°C had the 6-week-long shelf life, which is reduced to 3 weeks, if products are kept at 20°C.

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