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# BEHAVIOUR OF CLOSTRIDIAL SPORES DURING A MODEL FISH PRESERVATION AND AT ABUSE TEMPERATURE

(With 5 Tables)

By

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# سلوك جرثومات ميكروب الكلوستردیوم أثناء نموذج لحفظ الأسماك وفي درجة حرارة تعسفية

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تعتبر ميكروبات الكلوستردیوم بأنواعها المختلفة ذات أهمية قصوى في تلوث وتسمم الغذاء. وجرثومات هذه الميكروبات تقاوم المواد الحافظة والحرارة المستخدمة لحفظ الأغذية. في هذه الدراسة تم بيان تأثير محاليل حافظة كيميائية وطرق أخرى للحفظ على نمو جرثومات ميكروب الكلورستردیوم بيرفرنجنز DSM (٧٦٥) ، والكلوستردیوم بيرفرنجنز DSM (٧٩٨) وكذلك الكلوستردیوم بايفرمنتز . كانت المحاليل الحافظة المستخدمة ثلاثة أنواع هي:

(١) محلوم الملح المتبل ١٥٪ مع أسكوربات الصوديوم ٠,٥٪ ،

(٢) محلول الملح المتبل ٢٥٪ مع أسكوربات الصوديوم ٠,٥٪ ،

(٣) محلول كلوريد الصوديوم (ملح الطعام) ١٥٪ مع سوربات البوتاسيوم ٠,٥٪ وبنزوات الصوديوم ٠,٢٥٪ ، حمض الأسكوربيك ٠,٥٪ ، حامض الأسيتيك ٠,١٪ . وكانت الطرق الأخرى المستخدمة في الحفظ هي النزع الجزئي للماء والتعبئة تحت خلخلة الهواء . أثبتت النتائج أن المحلول الحافظ الثالث كان ذو كفائة عالية من المحاليل الأخرى (الأول والثاني) في منع نمو جراثيم الكلورستردیوم المختلفة بالإتحاد مع الطرق المستخدمة في الحفظ. كذلك كانت تأثير المحاليل المختلفة محل الدراسة مع الطرق الأخرى للحفظ متساوية في منع نمو جراثيم الكلوستردیوم في نهاية الأسبوع الرابع . تم تقدير متبقيات المواد الحافظة من نترات ونيتريت الصوديوم وكلوريد الصوديوم والسوربات والبنزوات وكذلك المعامل الهيدروجيني والنشاط المائي.

## SUMMARY

Behaviour of some Clostridial spores during a model fish preservation and at abuse temperature was investigated. The effects of various chemical

preservative solutions with other methods of fish preservation on the outgrowth of *C.perfringens* DSM 765, *C.perfringens* DSM 798 and *C.bifermentans* spores were studied. The preservative solutions (PS) were (i) nitrite curing salt (15%) with sodium ascorbate (0.5%), (ii) nitrite curing salt (25%) with sodium ascorbate (0.5%), (iii) sodium chloride (15%); potassium sorbate (0.5%); sodium benzoate (0.25%); ascorbic acid (o.5%) and acetic acid (1.5%). The other preservative methods used were partial dehydration (PD) and vacuum packaging. The results indicated that solution (iii) was the most efficient and rapid preservative in preventing spore outgrowth. The responses of the examined spores to all of the investigated preservative solutions and subsequent methods were similar at end of the fourth week of storage. The residues of the sodium nitrite, sodium nitrate, benzoate, sorbate, sodium chloride as well as pH and water activity were determined.

***Key words: Clostridial spores - Fish presevation.***

## INTRODUCTION

The spore-forming anaerobes are widely distributed in the environment. They are commonly found in the faeces of man, animal and in a variety of foods particulary of animal origin.

Spores of *Clostridium perfringens* can survive cooking and slow cooling. During refrigerated storage the spores germinate into vegetative cells which multiply rapidly. Under optimal conditions *C. perfringens* has a short generation time, 10-12 min, (MEAD, 1969), and can increase to a number sufficient to initiate illness.

*Clostridium perfringens* is responsible for a considerable number of food poisoning outbreaks annualy. From 1985-1989, *C. perferingens* food poisoning constituted 5.4, 12.2, 21.2, 7, 7, 9.1, 18 and 8 percent of the total food poisoning outbreaks in Germany, Denemark, Finland, France, Iceland, Netherland, Swisserland and United Kingdom, respectively (ANON., 1990). Fish, shrimp and crab were incriminated in *C. perfringens* food poisoning

outbreaks (DUNCAN, 1974). The enterotoxin produced caused the symptoms of illness when the ingested cells sporulated in the intestines (CRAVEN, 1980). *Clostridium bifermentans* is a spoilage, proteolytic and an anaerobic microorganism. It could be isolated from meat, fish, shrimp sauce and spices .

Common carp (*Cyprinus carpio*) is a fresh water fish, grows fast under tropical climatic conditions. It is also sturdy fish able to withstand handling, high temperatures and salinties (GELMAN and BENJAMIN, 1989). The nutritive value of common carp was studied recently by STOLLE *et al.* (1994 b).

Tradionally, fish is preserved by the addition of salts. Curing in this way is mainly by the addition of common salt, and at the present time a combination of different ingredients are used. The permitted curing agents used are sodium chloride, sodium nitrite, ascorbic acid and others.

Common salt acts as a preservative and a flavouring agent. Nitrite is a colour fixative, has antioxidant and some bacteriostatic effects and it is added in the form of nitrite curing salt (ROBERTS, 1982). Benzoic acid has an inhibitory effect on food poisoning and spore forming bacteria (BAIRD-PARKER, 1980). Ascorbic acid accelerates and stabilizes the development of cured coloured pigment. It affects the anticlostridial activity of nitrite. Ascorbic acid acts as a reducing agent, through oxygen scavenger, consequently it is an antioxidant and decreases the redox potential of the substrate (ANON, 1980).

Furthermore, acetic acid in combination with other preservatives may stimulate the action of salt against pathogenic microorganisms specially clostridia (LUECK, 1986). The use of nitrite curing salt alone or with sodium ascorbate (NASSAR, 1993); sorbate (SOFOS and BUSTA, 1981); Benzoate (GELMAN *et al.* 1985) and with other combined methods of preservation were studied for inhibition of microorganisms and consequently increase the durability of the food product.

Factors influencing the growth and survival of *C. perfringens* are; the temperature, water activity , nutritional requirement and heat resistance. The spores have various degrees of resistance (HOLAND *et al.*, 1969 and ROBERTS, 1982).

Combination of different agents aids to lower the water activity of cured fish, while partial dehyderation leads to more lowering of water activity. Whereas, spores of clostridial species if present, are unable to germinate, grow or produce toxin.

The aim of this work was to study the behaviour of some Clostridial spores during a model fish preservation and storage at 25$^0$C.

## MATERIALS and METHODS

**A) Fish** :

Twenty fish *(Cyprinus carpio)* were filleted, each fillet of 50 g in weight.

**B) Clostridial strains:**

Different clostridial strains; *C.perfringens* DSM 756 and *C.perfringens* DSM 798 were obtained from German collection for microorganisms Institute (Kolumbach). *C.bifermentans* was isolated from shrimp sauce and obtained from Institute of Hygiene and Food technology, München, Germany.

**C)Spores** :

Different Clostridial strains were grown on Elner media as described by (ELLNER, 1965) and incubated at 37 $^0$C for 7 days anaerobicallay in tubes covered by a layer of mixed parafin (2 parts of Merck 7164 and 1 part of Merck 7162) and Tortora media (TORTORA, 1984) which incubated anaerobically in tubes at 37$^0$C for 24 h for sporulation. The spores were obtained after heating the tubes at 80 $^0$C for 10 min. in water bath to destroy the vegetative cells and to expell the dissolved oxygen, then cooled (EISGRUBER and REUTER, 1991, BOWLES and MILLER, 1994). The

spore suspension was made according to the method of STRONG *et al.*

**D)Inoculation of samples:**

1 ml spore suspension was inoculated into each fish fillet. The inocula of *C.bifermentans* spores were $0.9x10^3$, $2.6x10^5$, $6.3x10^3$ and $1.5x10^4$ ,and that of *C.perfringens* spores were $0.8x10^3$, 1X10, 1x10 and $5x10^5$ colony forming unit /g (CFU/g) of fish fillet preserved by solution i.

Fish fillets exposed to preservativion by solution ii were inoculated by *C. bifermentans* spores in number of $0.9x10^3$ and $2.6x10^5$ and by *C.perfringens* spores in a count of $0.8x10^3$ CFU/g.

*C.bifermentans* spores for fish fillets preserved by preservative solution iii were $1.1x10^5$ and $2.5x10^4$ CFU/g .

**E) Preservative solutions of fish fillets** :

The various chemical preservative solutions (PS) were: (i) nitrite curing salt 15% with sodium ascorbate 0.5% solution. (ii)nitrite curing salt 25% with sodium ascorbate 0.5% solution. (iii) sodium chloride 15%; potassium sorbate 0.5%; sodium benzoate 0.25%; ascorbic acid o.5% and acetic acid 1.5% solution.

**F) Processing and examination:**

Processing was done as described by by NASSAR (1993) and STOLLE *et al.*, (1994a). The examinations were done before and after inoculation, after preservation (PS), after partial dehydration(PD) aswell as after 2, 4 and 6 Weeks of storage at $25^0$C.

**G) Growth and enumeration media:**

Liver broth (Oxoid CM 77) was incubated at 37 $^0$C for 48 h (KELCH, 1955) while cooked meat media (Oxoid 82) was incubated at 20 $^0$C for 7 days (ROBERSTON, 1916). The liver broth and cooked meat media were used for maintanance the spore growth. For enumeration, Sulphite Cycloserine Azide agar (SCA) of EISGRUBER and REUTER (1991), was incubated at $37^0$C anaerobically by using Anaerobic Gas-Pak

(BBL.060626) with gas generating Kit (Anaerobic system BR 38) and anaerobic indicator (Oxoid BR 55) for 48 h.

**H) Confirmtion of closridial strain**

Gram-reaction, colony morphology, catalase and oxidase tests were used to identify Clostridium. More confirmation for *C.perfringens* was done using reverse CAMP test (Oxoid CM 261 +7% sheep blood) incubated at 37 $^0$C for 24 h (BENTLER, 1981) and acid phosphate reaction was done. For *C.bifermentans*; indole reaction was done as a confirmatory test (COWANS and STEEL, 1974).

**I) Estimation of chemical preservatives:**

1. Sodium chloride was estimated according to the method of ANON (1986).
2. Nitrite,nitrate and total nitrite were estimated using a Zeiss PM 6 spectrophotometer at 540 nm and the method of ANON (1986).
3. Sorbate and benzoate estimation were carried out according to ANON (1986) with the use of High liquid pressure chromatography (HPLC) LDC /Milton Roy.
4. Hydrogen ion concentration (pH) was carried out as cited in ANON (1986) using equilibrated pH meter.
5. Water activity ($a_w$) was measured according to ROEDEL *et al.* (1979) using a Humidat TH2, Novasina AG, Zürich, Swiss.

## RESULTS

Results are presented in Tables 1, 2, 3, 4 & 5.

## DISCUSSION

All fresh fish fillets used in this study contained <10 (no detectable count) of Clostridial spores. Different inocula of spores were used in this study for many reasons; few information about what count can resist or rapidly destroyed by different presrvatives and during storage time; small umber of spores population may found in light contamination while large

numbers may found in heavy contamination, as well as, the spore count play an important role in spoilage, toxin production and /or interact with other factors ($a_w$, pH, Nitrite,..).

**Effect of preservative sol. i, partial dehydration and vacuum package on the clostridial spores at abuse temperature:**

Results presented in Table (1) indicated that the number of *C. bifermentans* decreased from $0,9x10^3$, $2.6x10^5$, $6,3x10^3$ and $1.5x10^4$ to <10, $1x10^2$, $1.4x10^4$ and $1x10^2$ CFU/g, respectively after removal of fishfillets from the preservative solution i by end of 46h while count of *C. perfringens* changed from $0.8x10^3$, 1x10, 1x10 and $5x10^5$ to 1x10, $1.7x10^2$, $8.8x10^2$ and $10^3$ CFU/g respectively after the same period in preservative solution i as shown in (Table 1). These fillets after this step had 263.4 ppm sodium nitrite ($NaNo_2$), 35.2 ppm nitrate ($KNO_3$) and 287.4 ppm total nitrite (Table 2). This level of nitrite together with pH 6.1 and $a_w$ 0.91 were slightly inhibitor for *C. bifermentans*. The inhibition of clostridial spp. spores by nitrite only was studied by some authors (LABBE and DUNCAN, 1970; ROBERTS and SMART, 1974). The effect of 156 ug of sodium nitrite per gram on the growth of *C. sporogens* spores in ground beef at abuse temperature was recently studied by BOWLES and MILLER (1994). After partial dehydration *C. bifermentans* had been <10, 1.5x10, $2x10^3$ and $1x10^2$ CFU/g , while the number of *C. perfringens* became 1.5x10, <10, <10 and $8.3x10^3$ CFU/g. These fish fillets had 4.1 pH and <0.70 $a_w$. The effect of water activity on the growth of *C. perfringens* was studied in culture media by STRONG *et al.* (1970) . These agents could play a major role in inhibiting spores present initially in the inoculated fish fillets, although some spores were likely to be viable for sometime. DUNCAN (1970) studied the variation of spore inocula and indicated that the spore concentration was considered an important factor in the spoilage of cured meats when nitrite inhibition of vegetative cells was being studied. The difference in inocula levels may explain many of the discrepancies in the litrature concerning the nitrite tolerance microorganisms under similar conditions (PERIGO and

ROBERTS, 1968). The effect of sodium nitrite, sodium chloride and sodium nitrate on germination and outgrowth of spores was studied by DUNKAN and FOSTER (1968). Generally nitrite effect was found to be greater under vacuum packaging than under aerobic conditions (LABOTS, 1977). After four weeks of storage at $25^0$C, Clostridial spores of different strains could not be detected. This could be attributed to the interactions between 4.1 pH, 0.66 $a_w$, 65.7 ppm residual nitrite and 11.3 g/100g sodium chloride,as well as, the storage temperature.

**Effect of preservative sol. ii, partial dehydration and vacuum package on clostridial spores at abuse storage.**

Preservation of fish fillets by the preservative solution ii previously inoculated by different inocula of different clostridia strains spores as in Tab. (3)., revealed that after two weeks of storage, the spores count decreased to 1.5x10 or <10 CFU/g. Regarding the two different species, spores of *C. bifermentans* was the better survival species in this experiment. TOMPKIN *et al.*, (1979) stated that a reaction between nitric oxide formed from nitrite and an essential iron of a cidophore involved in electron transport in clostridia, may account for the anticlostridial action. They suggested that nitric oxide-iron reaction product was reversible but stable as long as a reserve of nitrite was present.

**Effect of presrvative sol. iii, partial dehydration and vacuum package on Clostridial spores at abuse storage**

The preservation of fish fillets by the preservative solution iii resulted in a decrease in the number of *C. bifermentans* spores from $1.1x10^5$ and $2.5x10^4$ to $1.4x10^4$ and $1.2x10^3$ CFU/g respectively after removal from the preservative solution (Table 4). In this step, the fish fillets had a pH of 4.1 and 0,91 $a_w$. The count decreased to <10 after partial dehydration and remained constant up to 6 weeks of storage at $25^0$C. The preserved fish had 14.4 g/100 g sodium chloride, 7.1 g/kg benzoate and 4.1 g/kg sorbate (Table 5). From these findings, it is concluded that the use of preservative solution iii (sodium chloride, sodium benzoate, potassium sorbate, ascorbic acid and

acetic acid) was the most lethal one for Clostridial spores. Sorbate dissipated the proton motive force of the membrane and inhibited phenylalanine uptake, decrease the rate of protein synthesis, and altered patterns of phosphorylated nucleotides accumulation, which result in increasing the cellular concentration of glutamic pyruvic transaminase in putrefactive anaerobes 3679 (RONNING and FRANK, 1987). The inhibition of putrefactive anaerobes 3679 by sorbate may be due to a string-type response induced by protonophiric activity of sorbate. SOFOS (1989) mentioned that FAO (Food and Agriculture Organization) acceptable daily intake of sorbic acid and its salt is 25 mg/kg of body weight, which is the highest acceptable daily intake of the common food preservatives. In U.S.A., sorbic acid and sorbate are considered GRAS (generally ercogenised as safe compound). The maximum permissible limit of sorbic acid is usually between 0.1 and 0.2 % . The use of potassium sorbate was found to be effective as anticlostridial factor and consequently reduce the public health hazard of ntrite and this comply with TOMPKIN *et al.* (1974).

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TABLE 1: BEHAVIOUR OF *C.BIFERMENTANS* AND *C.PERFRINGENS* SPORES DURING FISH PRESERVATION BY PRESERVATIVE SOL.I AT ABUSE TEMPERATURE*

| Spore Type | ID | AP | PD | 2W | 4W | 6W |
|---|---|---|---|---|---|---|
| *C.bifermentans* | $0.9x10^3$ | <10 | <10 | <10 | <10 | <10 |
| ,, ,, | $2.6x10^5$ | $1x10^2$ | 1.5x10 | <10 | <10 | <10 |
| ,, ,, | $6.3x10^3$ | $1.4x10^4$ | $2x10^3$ | 5x10 | <10 | <10 |
| ,, ,, | $1.5x10^4$ | $1x10^2$ | $1x10^2$ | <10 | <10 | <10 |
| *C.perfringens*+ | $0.8x10^3$ | 1x10 | 1.5x10 | <10 | <10 | <10 |
| ,, ,, ++ | 1x10 | $1.7x10^2$ | <10 | <10 | <10 | <10 |
| ,, ,, + | 1x10 | $8.8x10^2$ | <10 | <10 | <10 | <10 |
| ,, ,, ++ | $5x10^5$ | $1x10^3$ | $8.3x10^3$ | <10 | <10 | <10 |

I.D:Inoculation dose CFU/g, AP: after 46 h in the preservative solution ; PD: after partial dehydration(35 $^0$C for 46 h), 2W-6W. storage weeks at 25 $^0$C.; +: DSM 798; ++: DSM 756.

TABLE 2. PRESERVATIVES, PH AND $A_W$ IN PRESERVED FISH BY PRESRVATIVE SOL. I:

| Preservative agents | After (48 hr) Preservation | After 4 weeks of storage | After 6 weeks of storage |
|---|---|---|---|
| Sod. chloride (g/100g) | 8.8 | 11.3 | 11.3 |
| $NaNO_2$ (pmm) | 263.4 | 4.1 | 4.1 |
| $KNO_3$ (pmm) | 35.2 | 90.2 | 90.2 |
| Total $NaNO_2$ (ppm) | 287.4 | 65.7 | 65.7 |
| pH | 6.1 | 4.1 | 4.1 |
| $a_w$ | 0.91 | 0.66 | 0.66 |

TABLE 3. BEHAVIOUR OF CLOSTRIDIAL SPORES DURING FISH PRESERVATION BY PRESERVATIVE SOL. II AT ABUSE TEMPERATURE

| Spore Type | ID | AP | PD | 2W | 4W | 6W |
|---|---|---|---|---|---|---|
| *C.bifermentans* | $0.9x10^3$ | $1.5x10^5$ | $1.1x10^5$ | <10 | <10 | <10 |
| ,, ,, | $2.6x10^5$ | $1x10^2$ | <10 | 1.5x10 | <10 | <10 |
| *C.perfringens*+ | $0.8x10^5$ | 1x10 | $8.3x10^3$ | <10 | <10 | <10 |

* Legened as below Table (1)

TABLE 4. BEHAVIOUR OF C. BIFERMENTANS SPORES DURING FISH PRESERVATION BY PRESERVATIVE SOL. III AT ABUSE TEMPERATURE

| Spore Type | ID | AP | PD | 2W | 4W | 6W |
|---|---|---|---|---|---|---|
| *C.bifermentans* | $1.1x10^5$ | $1.4x10^4$ | <10 | <10 | <10 | <10 |
| ,, ,, | $2.5x10^4$ | $1.2x10^3$ | <10 | <10 | <10 | <10 |

*Legened as below Table (1)

TAB. 5. PRESERVATIVES, PH AND $A_W$ IN FISH PRESERVED BY PRESERVATIVE SOL. (III)

| Preservative agents | After(48 hr) preservation | After 4 weeks | After 6 weeks |
|---|---|---|---|
| Sod. Chloride g/100 g | 7.2 | 14.4 | 14.4 |
| Benzoate g/kg | 5.3 | 7.1 | 7.1 |
| Sorbate g/kg | 4.4 | 4.1 | 4.1 |
| pH | 4.1 | 4.4 | 4.4 |
| $a_w$ | 0.91 | 0.66 | 0.66 |