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ANTIMICROBIAL EFFECTS OF SODIUM BENZOATE, SODIUM NITRITE AND POTASSIUM SORBATE AND THEIR SYNERGISTIC ACTION *IN VITRO*

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Abstract

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The aim of present work was to investigate the antimicrobial effects of sodium benzoate, sodium nitrite and potassium sorbate and their synergistic action (sodium nitrite + sodium benzoate, sodium nitrite + potassium sorbate, sodium benzoate + potassium sorbate) on selected food- spoiling bacteria and fungi, for a potential use in food industry. The following species of microorganisms were tested: *Bacillus subtilis*, *Bacillus mycoides*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Aspergillus flavus*, *Fusarium oxysporum*, *Candida albicans*, *Trichoderma harsianum* and *Penicillium italicum*. The strongest antimicrobial effect was exerted by sodium nitrite (MIC 0.5 mg/ml) in relation to the species *Pseudomonas aeruginosa*. Synergistic action was noticed against 40% of the tested species (*Escherichia coli*, *Staphylococcus aureus*, *Bacillus mucoides* and *Candida albicans*) in the case of the sodium nitrite + sodium benzoate combination; and against 30% of them (*Bacillus mucoides*, *Pseudomonas aeruginosa* and *Escherichia coli*) in that of the sodium nitrite + potassium sorbate combination. *Escherichia coli* manifested the greatest sensitivity to the combined action of preservatives, *Aspergillus flavus* the greatest resistance.

Key words: food-spoilage microorganisms, antimicrobial activity, preservatives, sodium benzoate, sodium nitrite, potassium sorbate, MIC, synergism

Introduction

Food can contain a variety of microorganisms such as the bacteria the yeasts and moulds witch have been reported as the causal agents of food borne diseases and/or food spoilage (Betts et al., 1999). The presence of microorganisms in foodstuffs can affect both the safety and quality of the product. Consequently, food manufacturers have developed food processing treatments that help preserve foods, by destroying the microorganisms that are present or by injuring them and thus preventing their growth. There are many sites within a bacterial cell that can become damaged when the bacteria are subjected to these food processing treatments. These sites include the genetic material of the cell (DNA, RNA) and also the cell membrane

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(Everis, 2001).

Different kinds of preservatives are used to prevent biodeterioration of food products. In the food industry, sodium benzoate, potassium sorbate and sodium nitrite are often used as preservatives. Sodium benzoate is a preservative that is widely used in the food industry. It is used as an antifungal agent (Salkowski, 1875) to conserve margarine, fresh juices, and sweets. European Commission limits for benzoic acid and sodium benzoate are 0.015–0.5% (EC, 1995).

Potassium sorbate is used to conserve cheeses, cakes and syrups. The effective concentration is 0.2% (Sofos and Busta, 1981). Sodium nitrite is used to conserve fish and meat products. Its effective concentration is 0.02 % (Noel et al., 1990).

The inhibitory effect of preservatives varies, depending on the concentration and type of preservative, pH of the medium and the species of microorganism. Inhibition of growth and activity of microorganisms can be achieved by lowering pH of the medium or by increasing the concentration of preservatives (Restaino et al., 1981). The effectiveness of use of preservatives is often dictated by limitations in their action or by sensitivity of the microorganisms themselves (Stickler and Tomas, 1982).

The objectives of the present work were to establish the antimicrobial effects of sodium benzoate, sodium nitrite and potassium sorbate, estimate the efficiency of their combined action against selected food spoilers and thereby expand the possibilities for more effective conservation of food.

Material and Methods

The preservatives used in the experiment were as follows: sodium benzoate (C Product, Belgrade, 2007); sodium nitrite (Laboratory of Biochemistry, Science Faculty, University of Kragujevac); and potassium sorbate (C Product, Belgrade, 2007). Different concentrations of preservatives were created by dissolving them in liquid Mueller-Hinton broth (Torlak, Belgrade). Before testing, preservatives were sterilized at 80°C for 10 min.

The antimicrobial activity of preservatives was tested in relation to the bacteria *Bacillus subtilis* (PMFKg-B1), *Bacillus mucoides* (PMFKg-B2), *Staphylococcus aureus* (PMFKg-B30), *Escherichia coli* (PMFKg-B12) and *Pseudomonas aeruginosa* (PMFKg-B40); and the fungi *Aspergillus flavus* (PMFKg-F5), *Fusarium oxysporum* (PMFKg-F18), *Candida albicans* (PMFKg-F8), *Trichoderma harsianum* (PMFKg-F6) and *Penicillium italicum* (PMFKg-F15).

All microorganisms were obtained from stock cultures of the Laboratory of Microbiology. Prior to testing, fresh bacterial cultures grew on an MPA substrate (27°C/24 h) (*Escherichia coli* grew on Endo agar at 37°C for 24 h), while fungal cultures grew on Sabourad's dextrose agar for 7 days at 22°C (*Candida albicans* at 37°C). Suspensions of bacterial and fungal spores were made in sterile water, density being determined spectrophotometrically and adjusted to 0.5 units of the McFarland scale (1-2Ч10^8 CFU/ml).

The minimal inhibitory concentration was defined as the lowest concentration of an antimicrobial agent at which there was no visible growth of the microbe. Minimal inhibitory concentrations were determined by the tube dilution method (NCCLS, 1997). The final concentrations of preservatives were as follows: for sodium benzoate — from 20 to 0.78 mg/ml for bacteria and from 50 to 0.5 mg/ml for fungi; for sodium nitrite — from 1 to 0.1 mg/ml for bacteria and from 50 to 0.5 mg/ml for fungi); and for potassium sorbate — from 20 to 0.2 mg/ml for bacteria and from 50 to 0.5 mg/ml for fungi. Each test tube was given 0.1 ml of the fabricated inoculums to an average density of 10 CFU/ml. The test tubes were incubated at 27°C for 24 h for bacteria (37°C in the cases of *Staphylococcus aureus* and *Escherichia coli*); and at 22°C for 48 h for fungi (37°C in the case of *Candida albicans*).

Synergism of the preservatives was assessed by the checkerboard assay method. The following combinations of preservatives were tested: sodium benzoate + sodium nitrite, sodium benzoate + potassium sorbate and potassium sorbate + sodium nitrite. From

the first to sixth horizontal column, the first preservative of the combination was doubly diluted in Mueller-Hinton broth (MIC values of up to 32), while the second preservative of the combination was doubly diluted (MIC values of up to 32) and added in a quantity of 0.1 ml from the first to sixth vertical row. The synergism of preservatives was determined by calculating the fractional inhibitory index according to the formula: FIC = FICA + FIC B = [A]/MIC_A + [B]/MIC_B. Types of effects were classified as follows: FIC J0.5, synergism; FIC 0.5-1, additive effect; FIC 1-4, indifferent effect; and FIC >4, antagonism. (Berenbaum 1981)

Results and Discussion

The minimal inhibitory concentration varied, depending on the kind of preservative and taxonomic characteristics of the species of microorganism tested. The results are presented in Table 1.

All of the tested preservatives exerted an antimicrobial effect, antifungal action requiring higher concentrations of the preservatives than antibacterial action.

The strongest antifungal activity was manifested by sodium benzoate (2.5 mg/ml) against the species *Candida albicans*. The lowest MIC values of potassium sorbate and sodium nitrite were 3.25 and 7.5 mg/ml, respectively, against the species *Fusarium oxysporum*. The most resistant of the fungi was *Aspergillus flavus*, MIC values for all three preservatives being greater than 50 mg/ml against it.

The strongest antibacterial activity was exhibited by sodium nitrite (MIC = 0.5 mg/ml) against the species *Pseudomonas aeruginosa*. The MIC of potassium sorbate was 5 mg/ml against *Escherichia coli*, while that of sodium benzoate was 5 mg/ml against *Escherichia coli* and *Pseudomonas aeruginosa.* Table 1 presents the results of testing the synergistic action of different combinations of preservatives. Synergistic action was established for the sodium nitrite + sodium benzoate and sodium nitrite + potassium sorbate combinations. Synergism was established at preservative concentrations corresponding to 1/4 MIC and lower. The sodium nitrite + sodium benzoate combination exhibited synergism in relation to the bacterial species *Escherichia coli*, *Staphylococcus aureus* and *Bacillus mycoides* and the fungal species *Candida albicans*. Synergism was quantitatively represented by the FIC index, which for this combination ranged from 0.31 to 0.5. MIC values of sodium nitrite and sodium benzoate were reduced in present work up to three times (synergism established at j MIC values). The sodium nitrite + potassium sorbate

Table 1
MIC values of preservatives and FIC index of different combination of preservatives

Species	MIC, mg/ml			? FIC index		
	S.benz.	S.nitrite	P.sorbate	S.benz.+ S.nitrite	S.nitrite + P.sorbate	P.sorbate + S.benz.
Bacillus mucoides	10	2	10	0.31(S)	0.28(S)	2.0(I)
Bacillus subtilis	10	1	10	1.25(I)	0.75(A)	1.5(I)
Staphylococcus a.	10	1	10	0.375(S)	1.125(I)	1.25(I)
Pseudomonas aer.	5	0.5	10	1.5(I)	0.375(S)	2.0(I)
Escherichia coli	5	2	5	0.31(S)	0.28(S)	0.65(A)
Aspergillus flavus	50	50	50	0.75(A)	2.0(I)	0.75(A)
Candida albicans	2.5	50	50	0.375(S)	0.75(A)	2.0(I)
Fusarium oxy.	20	3.25	7.25	1.5(I)	2.0(I)	1.5(I)
Trichoderma hars	30	50	30	1.5(I)	1.5(I)	2.0(I)
Penicillium italicum	20	50	15	0.75(A)	1.5(I)	2.0(I)

combination exhibited synergism in relation to the bacteria *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus mycoides*. The FIC index fluctuated from 0.28 to 0.5. MIC values sodium nitrite and potassium sorbate were reduced in present work up to four times (synergism established at range j MIC- 1/8 MIC values). The potassium sorbate + sodium benzoate combination did not manifest synergism in relation to the selected species.

The sodium nitrite + sodium benzoate combination exhibited a synergistic effect against 4 out of 10^4 species (40%), viz., *Escherichia coli*, *Staphylococcus aureus*, *Bacillus mucoides* and *Candida albicans*; an additive effect against 3 out of 10 species (30%), viz., *Aspergillus flavus*, *Trichoderma harsianum* and *Penicillium italicum*; and an indifferent effect against 3 out of 10 species (30%), viz., *Bacillus subtilis*, *Pseudomonas aeruginosa*. The sodium nitrite + potassium sorbate combination exhibited a synergistic effect against 3 out of 10 species (30%), viz., *Escherichia coli, Bacillus mucoides* and *Pseudomonas aeruginosa*; an additive effect against 2 out of 10 species (20%), viz., *Candida albicans*, and *Bacillus subtilis*; and an indifferent effect against 5 out of 10 species (50%), viz., *Staphylococcus aureus*, *Aspergillus flavus*, *Fusarium oxysporum*, *Trichoderma harsianum* and *Penicillium italicum*. The sodium benzoate + potassium sorbate combination exhibited no synergistic effect; an additive effect against 2 out of 10 species (20%), viz., *Aspergillus flavus* and *Escherichia coli*; and an indifferent effect against 8 out of 10 species (80%), viz., *Bacillus subtilis*, *Bacillus mucoides*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Fusarium oxysporum*, *Candida albicans*, *Trichoderma harsianum* and *Penicillium italicum*.

Some microorganisms have developed ways to survive some processing treatments. These include the production of heat shock and cold shock proteins that help the cell function normally under higher or lower temperatures than normal. Some treatments will cause irreparable damage and the cells will be destroyed. However, sometimes the damage will be repairable and the cells are able to repair and recover. The micro-organisms that are destroyed by processing will not cause subsequent food poisoning or spoilage, but organisms that are injured and become repaired could cause subsequent food spoilage or poisoning (Everis, 2001).

In the present work, the tested microorganisms exhibited resistance to lower concentrations of the selected preservatives. The MIC values of sodium benzoate, sodium nitrite and potassium sorbate were higher than the recommended effective concentrations. We therefore tested the effectiveness of synergistic action of the preservatives on selected microorganisms that cause biodeterioration of food.

It is known that the effectiveness of individual or combined action of preservatives depends not only on their concentration and type, but also on the species of microorganism on which they act. In the present work, the sodium nitrite + potassium sorbate and sodium nitrite + sodium benzoate combinations of preservatives exhibited synergistic and additive effects. The greatest sensitivity to these combinations was manifested by *Escherichia coli*, *Staphylococcus aureus* and *Bacillus mucoides*. *Aspergillus flavus*, *Fusarium oxysporum, Penicillium italicum and Trichoderma harsianum* were the species most resistant to all three combinations of preservatives.

Conclusion

The results of this study demonstrate more effective antimicrobial action of preservatives when used in combination with other preservatives than when used individually, a finding that can contribute to more effective conservation of food. Synergism was recorded at MIC values of 4 and lower, which indicates the possibility of avoiding the use of higher concentrations of sodium nitrite, sodium benzoate, and potassium sorbate that could lead to accumulation of toxic products in conserved food.

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