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ANTIBACTERIAL EFFECTS OF COMMERCIALLY AVAILABLE PHOSPHATES ON SELECTED MICROORGANISMS

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Abstract

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In the food industry, phosphates, polyphosphates and their salts are used, for example, as emulsifying agents in the production of processed cheese. The inhibitory effects of three commercially available phosphates and polyphosphates differing in their chain length (690, S9 and HBS) were tested on a set of 15 gram-positive or gram-negative CCM (Czech Collection of Microorganisms) strains and on 12 bacterial strains isolated from processed cheeses. Five different concentrations of each phosphate were chosen (0.1, 0.2, 0.3, 0.4 and 0.5% w/v) in order to observe the inhibitory effects of the phosphate salts on the growth of the microorganisms tested. Sensitivity of the individual bacterial strains to phosphates was observed of a liquid cultivation medium which was supplemented with applied salts. Subsequently, the growth in cells was determined by measuring optical density at a wavelength of 600 nm. According to the results, 690 and S9 phosphates, containing mainly orthophosphates, diphosphates (pyrophosphates) and short-chain polyphosphates, do not have a significant inhibitory effect on the growth of the tested bacteria. Significant inhibitory effects were observed only in HBS salt (a mixture of long-chain polyphosphates), which showed antibacterial effects on all gram-positive bacteria tested (both the CCM strains and those isolated from processed cheeses). The antibacterial effect of phosphates on gram-positive microorganisms is growing with the increasing length of the polyphosphate chain. This study has not proved a significant effect of the phosphates tested on the growth of gram-negative bacteria used.

phosphate, polyphosphate, microorganisms, antibacterial effect

In the production of food, phosphates are frequently used as additives in order to improve technological and functional qualities of foodstuffs. In particular, phosphates significantly influence the properties of proteins present in food. Their effect is connected mainly with modification of the environmental conditions in which they can cause a change in pH or ion strength of a solution. Apart from the above-mentioned properties, phosphates have the ability to bind water and chelate cations. The ability to chelate is influenced, for example, by the number of monomers in a molecule (with the rising number of phosphates the affinity with cations increases), a particular metal cation, temperature, pH etc. Moreover, phosphates can also influence the formation of gel or have an inhibitory effect on the growth of microorganisms (Molins, 1991; Guinee *et al.*, 2004; Mizuno and Lucey, 2005).

Antimicrobial effects of phosphates are described mainly in gram-positive bacteria, some micromycets and yeast cells (Knabel *et al.*, 1991; Zaika and Kim, 1993; Lee *et al.*, 1994a; Loessner *et al.*, 1997; Suarez *et al.*, 2005, 2007). The inhibitory effect on gram-positive bacteria is dependent on the chain length (level of condensation) of phosphates. Long-chain phosphates have better inhibitory effects than short-chain phosphates. Also, the inhibitory effect can be influenced by temperature and pH (higher sensitivity when pH > 7.4), the initial population of microorganisms or the addition of metal ions (Jen and Shelef, 1986; Lee *et al.*, 1994a; Zaika *et al.*, 1997; Maier *et al.*, 1999). The effect on long-chain polyphosphates

works according to the principle of chelation of mainly divalent metal ions (Ca^{2+} a Mg^{2+}), which are essential for maintaining the integrity of cell walls in gram-positive bacteria by forming transverse bridges between the molecules of teichoic acid in a cell wall (Lee *et al*., 1994b). Chelation of divalent ions can also cause these ions to be inactive to some essential physiological processes of growth. It has been found out that the protein responsible for the formation of septum during cell division (FtsZ protein) has a GTP activity, which is strictly dependent on the presence of magnesium ions (Maier *et al*., 1999). An elimination of the above-mentioned ions results in a bactericidal and bacteriolytic effect (Lee *et al*., 1994b; Maier *et al*., 1999). Moreover, in spore-forming bacteria, polyphosphates have an inhibitory effect on spore germination (Eckner *et al*., 1994; Loessner *et al*., 1997; Maier *et al*., 1999; Varga, 2005; Borch and Lycken, 2007). In *Bacillus cereus,* the effect of phosphates on the morphology of cells growing in an exponential phase has been described. It leads to lysis of the cells and the inability to form septum during their division. Sublethal concentrations of polyphosphates in this bacteria cause significant lengthening of their cells, which can even be in the shape of fibres (Maier *et al*., 1999). The inhibitory effect of polyphosphates on microorganisms can be reduced by adding multivalent metal ions to the cultivation medium (Jen and Shelef, 1986; Lee *et al*., 1994b; Zaika *et al*., 1997; Maier *et al*., 1999). The effects of phosphates on gram-negative bacteria are described in literature very rarely. In laboratory conditions, the inhibitory effect on *Aeromonas hydrophila* has been determined (Velazquez *et al*., 2001).

Apart from observing the effect of polyphosphates on microorganisms in laboratory conditions, their influence on microorganisms in real foodstuffs has also been studied. Molins *et al.* (1985) found out that the addition of phosphates can decrease the number of bacteria *Clostridium sporogenes* on meat products kept in storage. Suarez *et al.* (2005) studied the inhibitory effect of commercially available phosphates on moulds (*Byssochlamys nivea, Aureobasidium pullulans* and *Penicillium glabrum*) isolated from the food processing industries. Inhibitory effects of polyphosphates on bacteria which can spoil dairy products, especially processed cheese spreads, are also described in literature. The addition of polyphosphates to these products can reduce or stop the growth of undesirable spore-forming bacteria (especially clostridia), which can participate in the spoiling process by producing gas, butyric acid or toxins (Briozzo *et al*., 1983; Eckner *et al*., 1994; Loessner *et al*., 1997; Varga, 2005; Borch and Lycken, 2007).

One of the products in which phosphates are used is processed cheese. During its production, phosphates and polyphosphates in the form of sodium salts are used as emulsifying salts. Emulsifying salts (the concentrations of which are usually 2–3% w/w) are added in order to ensure a smooth and homogenous structure of processed cheese without separating water, fat and proteins (Carić *et al*., 1985). Phosphates have the function of so called emulsifying agents, modifying the environment in the production of processed cheese so that the caseins present could function as emulsifiers. This can be reached mainly by splitting off calcium from acid amino acids and phosphoseryl residues of protein matrix and by means of peptization, hydratation and swelling of proteins or emulsification of fats (Guinee *et al*., 2004). The ability to chelate cations is also used in the production of processed cheese, where on the one hand, calcium ions are attracted from caseins to phosphates by greater electrostatic forces and on the other hand, sodium ions bind to casein (Molins, 1991; Guinee *et al*., 2004).

The aim of this study was to observe antimicrobial effects of three commercially available phosphates of different levels of condensation (different chain length) on CCM microorganisms and subsequently on bacteria isolated from long-stored processed cheeses to which phosphates are added in the form of emulsifying salts.

MATERIAL AND METHODS

In the initial phase, the effects of emulsifying salts on the growth of microorganisms were observed on collected bacteria gained from the Czech Collection of Microorganisms (CCM) and from the collection of Secondary School of Dairy Industry in Kroměříž (SPSM). In the second phase, the influence of emulsifying salts on bacteria isolated from processed cheeses was observed.

In the first phase of the experiment, bacteria which can contaminate foodstuffs or can be of clinical significance were chosen in order to determine the inhibitory spectrum of the emulsifying salts on microorganisms. There were chosen 7 gram-negative bacteria (*Escherichia coli* CCM 180, *Salmonella enterica* ser. Enteritidis CCM 4420, *Serratia marcescens* CCM 303, *Proteus mirabilis* CCM 7188, *Citrobacter freundii* CCM 7187, *Pseudomonas aeruginosa* CCM 3955 a *Flavobacterium* sp. SPSM 2901) and 8 gram-positive bacteria (*Staphylococcus aureus* subsp. *aureus* CCM 3953, *Micrococcus luteus* CCM 732, *Corynebacterium glutamicum* CCM 2428, *Bacillus cereus* CCM 2010, *B. subtilis* CCM 2216, *B. sphaericus* CCM 1615, *B. brevis* SPSM 4101 and *B. stearothermophilus* SPSM 4103).

In the second phase of the experiment, inhibitory effects of phosphate salts on bacteria isolated from processed cheeses stored at 6 ± 2°C for 12 months were tested. The processed cheeses were made from a mixture of natural cheeses, curd, butter, water and emulsifying salts of phosphates as part of the standard production by a cheese producer in the Czech Republic. The melt was poured into 75-gram containers made of drawn aluminium and decked with a sealable aluminium lid. The products contained 40% w/w dry matter and 45% w/w fat in dry matter. For the purpose of this experiment, from the isolated bacteria the following were chosen: gram-positive aerobic spore-forming rods, catalase positive (isolates NTS 01, NTS 02, NTS 03, NTS 04, NTS 05,

NTS 06) and gram-negative rods catalase positive, oxidase negative (isolates NTS 07, NTS 08, NTS 09, NTS 10, NTS 11, NTS 12, NTS 13).

In order to find out antibacterial effects of commercially available emulsifying salts of phosphates (sodium salts), the following were chosen: (i) HBS – a mixture of polyphosphates with a high level of condensation and less amount of orthophosphates; (ii) S9 – a mixture of polyphosphates (a lower level of polymerization than HBS) and orthophosphates; (iii) 690 – a mixture of orthophosphates and diphosphates. HBS and S9 are produced by BK Ladenburg GmbH, Germany; 690 is produced by Chemische Fabrik Budenheim, Germany.

In order to find out the sensitivity of the bacteria chosen to phosphate salts, 5 different concentrations of each phosphate were used (0.1; 0.2; 0.3; 0.4 and 0.5% w/v). The microorganisms were cultivated in nutrient broth. The phosphates of particular concentrations were added directly to the liquid cultivation medium (pH modified to 7.0 by means of 1M NaOH), which was subsequently autoclaved (15 min, 121°C). The media supplemented with particular phosphates were dispensed in 5-ml portions into test tubes and subsequently inoculated by 100 µl of overnight culture of the bacteria tested, diluted 1:100 (1,4–7,8·10^6 CFU/ml). The control samples contained nutrient broth with inoculated bacteria but without phosphate salts. The bacteria tested were cultivated for 24 hours at 30°C or 37°C (only *B. stearothermophilus* at 45°C), the bacterial strains *Bacillus*, *Micrococcus* and gram-positive isolates from processed cheeses were cultivated while being shaken. The growth of microorganisms was determined spectrophotometrically by measuring the optical density of the cells at a wavelength of 600 nm (OD_{600}) in comparison with nutrient broth without inoculated cells on a spectrophotometer with a diode array (LIBRA S6 Biochrom, England).

RESULTS AND DISCUSSION

The effect of three commercially available phosphate salts with different levels of condensation (chain length) was tested on 28 bacterial strains by means of spectrophotometrical analysis of the growth of bacteria measured at a wavelength of 600 nm. Measuring optical density of cells at 600 nm is a suitable method for quick determination of an inhibitory effect of a particular substance (Lee *et al.*, 2002) – commercially available phosphate salts in our case, on microorganisms. The minimum inhibitory concentration was in this case defined as the amount of phosphate needed to reduce the optical density of bacteria bellow 0.075, which corresponds with the method presented in the work by Loessner *et al.* (1997).

As far as gram-negative bacteria are concerned, no significant inhibitory effect of the emulsifying salts used was found out (Loessner *et al.*, 1997). For this reason, Table I illustrates only the effects of the highest concentrations of the phosphates tested (0.5% w/v) on gram-negative bacteria. As it is clear from the results, even the highest concentrations of the phosphates tested did not have a significant inhibitory effect on the growth of gram-negative bacteria tested. As for gram-negative rods from the family *Enterobacteriaceae,* no significant growth inhibition took place. A more noticeable effect was observed

I: *Effect of emulsifying salts tested on gram-negative bacteria**

	Control (OD_{600})	0.5% HBS	0.5% S9	0.5% 690
Escherichia coli CCM 180	0.348	0	0	0
Serratia marcescens CCM 303	0.788	0	+	+
Salmonella Enteritidis CCM 4420	0.308	+	+	+
Proteus mirabilis CCM 7188	0.976	0	0	+
Citrobacter freundii CCM 7187	0.632	+	0	+
Pseudomonas aeruginosa CCM 3955	0.423	++	++	+
Flavobacterium sp. SPSM 2901	0.266	0	+	++
NTS 07	0.766	0	0	0
NTS 08	0.829	0	0	0
NTS 09	0.684	0	0	0
NTS 10	0.745	0	0	0
NTS 11	0.579	0	+	0
NTS 12	0.790	0	+	+
NTS 13	0.535	0	0	0

* Inhibitory effect of the substances tested is expressed by means of percentage of optical density (OD_{600}) of the inoculated broth containing emulsifying salt (of a given concentration) related to the value of optical density (OD_{600}) of a control sample without emulsifying salt inoculated by a given culture; $OD_{600} \leq 25$ % +++; 25% < $OD_{600} \leq 50$ % ++; 50% < $OD_{600} \leq 75$ % +; $OD_{600} > 75$ % **0** (no effect).

only in *Ps. aeruginosa* and *Flavobacterium* sp., which showed a decrease in the value of optical density of cells by ca 55% in comparison with the control. Also, the effects on gram-negative bacteria isolated from processed cheeses (probably members of family *Enterobacteriaceae*) were practically insignificant. The results presented are in accord with further studies (Knabel *et al.*, 1991; Loessner *et al.*, 1997), whose authors did not notice any inhibitory effect of emulsifying salts with polyphosphates on gram-negative bacteria either. Similarly, Vareltzis *et al.* (1997) did not find out any significant inhibitory effect of polyphosphate (in the form of sodium salt) on the inhibition of gram-negative bacteria contaminating the surface of poultry.

The inhibitory effect of commercially available phosphates on gram-positive bacteria is presented in Table II. As it is clear from the results, from the phosphate salts tested, only HBS salt showed a noticeable inhibitory effect. No significant inhibitory effect was noticed in S9 and 690 phosphates therefore only the highest concentrations of these two salts are presented in Table II.

II: *Inhibitory effect of the emulsifying salts tested on gram-positive bacteria**

	Control (OD_{600})	0.1% HBS	0.2% HBS	0.3% HBS	0.4% HBS	0.5% HBS	0.5% S9	0.5% 690
Staphylococcus aureus CCM 3953	0.325	+	+++	+++	+++	+++	+	0
Micrococcus luteus CCM 732	0.260	+	++	+++	+++	+++	+	+
Corynebacterium glutamicum CCM 2428	0.194	++	+++	+++	+++	+++	+	+
Bacillus cereus CCM 3953	0.412	+	+	+++	+++	+++	+	+
Bacillus subtilis CCM 2216	0.265	++	++	+++	+++	+++	0	0
Bacillus brevis SPSM 4101	0.316	+	++	+++	+++	+++	+	+
Bacillus sphaericus CCM 1615	0.448	+++	+++	+++	+++	+++	0	+
Bacillus stearothermophilus SPSM 4103	0.364	+++	+++	+++	+++	+++	+	+
Bacillus sp. NTS 01	0.211	++	+++	+++	+++	+++	0	0
Bacillus sp. NTS 02	0.231	0	0	+++	+++	+++	+	+
Bacillus sp. NTS 03	0.319	+	++	+++	+++	+++	0	+
Staphylococcus sp. NTS 04	0.301	++	+++	+++	+++	+++	+	0
Bacillus sp. NTS 05	0.251	0	0	+++	+++	+++	0	0
Bacillus sp. NTS 06	0.314	+	++	+++	+++	+++	0	0

* Inhibitory effect of the substances tested is evaluated by means of percentage of optical density (OD_{600}) of the inoculated broth containing emulsifying salt of a given concentration related to the value of optical density (OD_{600}) of a control sample without emulsifying salt inoculated by a given culture; $OD_{600} \leq 25\,\%$ and $OD_{600} \leq 0.075$ **+++**; $25\% < OD_{600} \leq 50\,\%$ **++**; $50\% < OD_{600} \leq 75\,\%$ **+**; $OD_{600} > 75\,\%$ **0** (no effect).

HBS phosphate salt at a concentration of 0.1% w/v was able to inhibit the growth of bacterial strains *Bacillus sphaericus* and *B. stearothermophilus*, which showed practically no growth in the presence of 0.1% HBS. As for bacterial strains *Staphylococcus aureus* and *Corynebacterium glutamicum*, the minimum inhibitory concentration of this salt was observed at 0.2% w/v. As far as the other gram-positive bacteria tested are concerned (*M. luteus, B. cereus, B. subtilis, B. brevis*), their growth was inhibited by the presence of HBS emulsifying salt at a concentration of 0.3% w/v. Similar conclusions were also reached by Loessner *et al.* (1997), who found out that 0.3% w/v HBS is able to inhibit the growth of the majority of gram-positive bacteria tested in laboratory conditions. Maier *et al.* (1999) tested the effects of HBS on the morphology of *B. cereus* and came to the conclusion that the concentrations of 0.1% and above have an inhibitory effect on the bacteria, which is demonstrated by cell lysis of the bacteria in the logarithmic phase of the growth. Other authors (Jen and Shelef, 1986; Lee *et al.*, 1994a) verified the effect of phosphates on bacterial strains *S. aureus* and found out that the inhibitory concentrations of these salts range between 0.1–0.5% w/v depending on the type of bacterial strain studied.

In gram-positive isolates from long-stored non-sterilized processed cheeses, the effect of HBS phosphate also caused the inhibition of growth of the bacteria. In the isolates from processed cheeses, the minimum inhibitory concentration of HBS salt was determined at 0.3% w/v with the exception of two isolates (NTS 01 and NTS 04), which were inhibited by a lower concentration (0.2% w/v). It is interesting to note the effect of HBS on two isolates, namely

NTS 02 and NTS 05. Lower concentrations (0.1 and 0.2% w/v) of the salt had minimum effects on the bacteria mentioned, while the concentration of 0.3% w/v had an inhibitory effect. Such behaviour of the bacteria can be caused by the fact that these strains had been isolated from processed cheeses and thus a better adaptation of the bacteria to the presence of phosphates can be assumed due to the addition of phosphate emulsifying salts, the lower concentrations of which could be tolerated. (ICMSF, 2005)

In literature, there have been published papers (Lee *et al.*, 1994a, 1994b; Loessner *et al.*, 1997; Maier et *al.*, 1999,) examining the inhibitory effects of phosphate salts with different levels of condensation on microorganisms. These authors came to the conclusion that the more polyphosphates with a high level of condensation the emulsifying salts contain, the higher their antibacterial effect is. Their conclusions are in accord with our results, which show that HBS emulsifying salt containing polyphosphates of the highest level of condensation had the most significant inhibitory effect on the bacteria tested.

In laboratory conditions, the values of minimum inhibitory concentration of HBS are relatively low. However, it can be assumed that in a complex matrix presented by foodstuffs (e.g. processed cheese or meat) the minimum concentration which will have an inhibitory effect on the microorganisms present (mainly gram-positive bacteria including spore-forming bacteria) will have to be increased. This hypothesis can be confirmed by the results of Loessner *et al.* (1997) and Varga (2005) where 0.5% is the minimum concentration of polyphosphate salts recommended in order to inhibit the growth of undesirable microflora in processed cheeses. The same conclusion concerning the necessity to apply a higher concentration of polyphosphates in order to inhibit microorganisms in real foodstuffs in comparison with those in laboratory conditions was described by Rajkowski *et al.* (1994) for *S. aureus* and *Listeria monocytogenes* in the environment of UHT-sterilized milk and by Lee *et al.* (1994a) for *S. aureus* in the environment of ground meat.

CONCLUSION

In laboratory conditions, it was found out that from the phosphate emulsifying salts, only HBS emulsifying salt containing a mixture of polyphosphates with a high level of condensation has significant antibacterial effects which occur solely on gram-positive bacteria tested. No inhibitory effect on gram-negative bacteria was observed. As far as S9 and 690 emulsifying salts are concerned, strict inhibition was not observed in any of the bacterial strains tested. Thus it can be said that the inhibitory effect of emulsifying salts on microorganisms is dependent on the condensation level of phosphates.

SOUHRN

Antibakteriální účinky komerčních fosfátů na vybrané mikroorganismy

Fosfáty, polyfosfáty a jejich soli se v potravinářském průmyslu využívají například při výrobě tavených sýrů jako tavicí soli. Inhibiční účinky tří komerčně využívaných fosfátů a polyfosfátů lišících se délkou řetězce (690, S9 a HBS) byly testovány na 15 sbírkových kmenech grampozitivních a gramnegativních bakterií a dále u 12 kmenů bakterií, které byly izolovány z tavených sýrů. Pro sledování inhibičního působení použitých fosfátových solí na růst testovaných mikroorganismů bylo zvoleno pět koncentrací každého fosfátu (0,1, 0,2, 0,3, 0,4 a 0,5 % w/v). Citlivost jednotlivých kmenů bakterií k fosfátům byla sledována v tekutém kultivačním médiu, které bylo obohaceno o příslušné soli a následně byl nárůst buněk zjišťován měřením optické denzity při vlnové délce 600 nm. Výsledky ukazují, že fosfáty 690 a S9, obsahující především orthofosfáty, difosfáty (pyrofosfáty) a polyfosfáty s krátkým řetězcem, nemají významný inhibiční vliv na růst testovaných bakterií. Signifikantní inhibiční účinky má pouze sůl HBS (směs polyfosfátů s dlouhým řetězcem), která vykazovala antibakteriální účinky vůči všem testovaným grampozitivním bakteriím (sbírkovým i izolovaným z tavených sýrů). Antibakteriální efekt fosfátů vůči grampozitivním mikroorganismům roste se zvyšující se délkou polyfosfátového řetězce. Tato studie neprokázala významný účinek testovaných fosfátů na růst použitých gramnegativních bakterií.

fosfáty, polyfosfáty, mikroorganizmy, antimikrobiální efekt

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