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THE INFLUENCE OF POLYPHOSPHATE MIXTURE ON THE GROWTH OF BACTERIA SIGNIFICANT IN THE FOOD INDUSTRY

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ABSTRACT

Phosphates are frequently used in the food processing, especially in the dairy and meat industries. They act as emulsifying salts, crucial in the manufacturing of processed cheeses. The effect of Cremosal AB4, a commercially used polyphosphate mixture, was tested *in vitro* on the growth of nine bacterial strains significant in the food industry: *Staphylococcus*, *Bacillus*, *Geobacillus*, *Clostridium*, *Enterococcus*, *Lactococcus*, *Lactobacillus*, and *Escherichia*. Five phosphate concentrations (0.1, 0.2, 0.3, 0.4, and 0.5% w/v, calculated on P_2O_5) were selected to assess the effect of the polyphosphate mixture on bacterial growth. The susceptibility of each bacterial strain to Cremosal AB4 phosphate was evaluated in a liquid culture medium enriched with the appropriate salt concentration. Subsequently, cell growth was assessed by measuring optical density at 850 nm. The results indicate that even at the lowest tested concentration of 0.1% w/v, Cremosal AB4 polyphosphate effectively inhibited the growth of all bacteria examined, except *Lactococcus lactis* subsp. *lactis* CCDM 141 and Escherichia coli CCM 3954.

Keywords: polyphosphate, Cremosal AB4, bacteria, inhibition effect

INTRODUCTION

Phosphates are often added to foodstuffs during the production in order to enhance their technological and functional properties. Phosphates in food significantly affect the properties of the proteins present. Their effect is primarily associated with modifying environments, where they can cause change in pH or ionic strength of a substance. Phosphates are commonly used in processed cheese, where sodium phosphates and polyphosphates serve as emulsifying agents. This helps create a smooth and homogeneous texture in the cheese, preventing the separation of water, fat, and proteins. A positive side effect of polyphosphate application can be seen in their antimicrobial effects **(Carić *et al.*, 1985; Molins, 1991**).
Phosphate salts inhibit the growth of bacteria, especially gram-positive ones, and some yeasts and fungi. This is associated with the structure of the cell wall and the specific capacity of polyphosphates to bind to divalent calcium and magnesium ions. (**Buňka and Buňková, 2009; Maier *et al.*, 1999**). The antibacterial effect primarily results from disruption of the cell wall integrity caused by chelation of calcium and magnesium ions. Concurrently, this leads to a loss of osmoregulation and compromises the selective permeability of the cytoplasmic membrane. Disruption of the cytoplasmic membrane permeability may lead to a decrease in metabolic functions resulting from substrate leakage. Phosphate salts may also prevent spore germination in gram-positive bacteria of *Bacillus* sp., *Clostridium* sp. and other representatives isolated from these genera (e.g., *Geobacillus* spp., *Alicyclobacillus* spp., *Lysinibacillus* spp., *Paenibacillus* spp., *Weizmania* spp., *Thermoanaerobacterium* spp., *Desulfotomaculum* spp.). These salts may also adversely affect the shape and formation of the septum within cell division (e.g. in *Bacillus cereus*). Due to the defective growth or impaired cell proliferation, the effect of phosphates is also manifested by reduction of bacterial colonies (**Maier *et al.*, 1999; Matsuoka *et al.*, 1995; Molins, 1991**). In gram-positive bacteria, the inhibitory effect is influenced by the phosphate chain length (condensation stage). Longer-chain phosphates exhibit more effective antimicrobial activity compared to their shorter-chain counterparts. (**Buňková *et al.*, 2008; Jang *et al.*, 2016; Loessner *et al.*, 1997; Lorencová *et al.*, 2012; Maier *et al.*, 1999; Moon *et al.*, 2019**). The antimicrobial effect of phosphates is also greatly affected by pH. The change in pH that is induced by the addition of phosphates may play a role in the sequestering ability. The principle of the effect of long-chain polyphosphates lies in the chelating of mainly bivalent metal cations (Ca^{2+} and Mg^{2+}), which are essential for maintaining the cell wall integrity of gram-positive bacteria by forming cross-bridges between teichoic acid molecules in the cell wall. Chelation of bivalent ions can render them unavailable for essential physiological growth processes. Moreover, the subsequent cleavage of these ions contributes to a bactericidal effect. (**Knabel *et al.*, 1991; Lee *et al.*, 1994b; Molins, 1991; Sampathkumar *et al.*, 2003**).
In addition to studying the effect of polyphosphates on microorganisms in *in vitro* conditions, their effects on microorganisms in real foodstuffs were also examined (**Moon *et al.*, 2021; Obritish *et al.*, 2008**). **Molins *et al.* (1985)** observed that the use of phosphates can reduce the number of *Clostridium sporogenes* bacteria in stored meat products, **Suarez *et al.* (2005)** evaluated the inhibitory effects of commercial phosphates on moulds (*Aureobasidium pullulans, Byssochlamys nivea* and *Penicillium glabrum*) found in food processing plants. The literature also describes the antimicrobial effects of polyphosphates on bacteria that can cause spoilage in dairy products, especially processed cheeses. The incorporation of polyphosphates into these foods can slow down or prevent the growth of undesirable spore-forming bacteria (especially clostridia), which can contribute to the spoilage of these foods by producing gas, butanoic acid, or toxins. (**Briozzo *et al.*, 1983; Eckner *et al.*, 1994; Loessner *et al.*, 1997; Varga, 2005; Borch and Lycken, 2007**).
The inhibitory effect of polyphosphates can be reduced by their hydrolysis, which can be caused, for example, by thermal heating. Some bacteria also have enzymes that are able to break down phosphate salts. However, most of them require magnesium, calcium or manganese ions for their activity, which leads to competitive behaviour, where enzymes and phosphates compete for the required alkali metal ions present (**Molins, 1991**).
The goal of our study was to monitor the effect of Cremosal AB4 phosphate mixture on the growth of nine bacterial strains significant in the food industry which can occur in processed cheeses.

MATERIAL AND METHODS

Tested microorganisms

The influence of phosphate mixture Cremosal AB4 was tested on several selected gram-positive bacterial strains sourced from the Czech Collection of

Microorganisms (CCM), the Cultures Collection of Dairy Microorganisms (CCDM) and the Collection of Animal Pathogenic Microorganisms (CAPM): *Staphylococcus aureus* subsp. *aureus* CCM 3953, *Enterococcus durans* CCDM 53, *Lactococcus lactis* subsp. *lactis* CCDM 141, *Lactobacillus helveticus* CCDM 142*Bacillus cereus* CCM 2010, *Geobacillus stearothermophilus* CCM 2062, *Clostridium butyricum* CAPM 6342 and *Clostridium sporogenes* CAPM 6329. The gram-negative bacterium *Escherichia col*i CCM 3954 was also used to compare the effect of this phosphate salt.

Solutions and chemicals

Polyphosphate salt mixture Cremosal AB4 (polyphosphate mixture consists of sodium hexametaphosphates – E452) was kindly provided by FOSFA plc., Břeclav, Czech Republic. A 10% (w/v) aqueous solution was prepared by dissolving an appropriate amount of salt in distilled water to evaluate its antibacterial properties. This solution was then sterilized through filtration (filter porosity 0.22 µm) and stored in sealed containers at room temperature.

Polyphosphate effect on bacterial growth

Five different concentrations of polyphosphate Cremosal AB4 (0.1, 0.2, 0.3, 0.4, 0.5 w/v; calculated to P_2O_5) were prepared to assess antimicrobial activity. The polyphosphate solutions were directly added to sterile culture media – Mueller Hinton Broth (HiMedia Laboratories, Mumbai, India; *S. aureus, B. cereus, G. stearothermophilus, Ent. durans, E. coli*), Reinforced Clostridial Broth (HiMedia Laboratories, Mumbai, India; *C. butyricum, C. sporogenes*) or de Man, Rogosa and Sharpe broth (HiMedia Laboratories, Mumbai, India; *L. lactis* subsp. *lactis, Lbc. helveticus*). Polyphosphate-containing media were dispensed in 30 ml aliquots into test tubes. These tubes were then inoculated with 100 µl of a 1:100 diluted overnight bacterial culture (1.6–6.8 × 10^6 CFU/ml). The control samples consisted of the inoculated broth, but without the polyphosphate addition.

Cell growth was monitored at 30±1°C (55 °C for *G. stearothermophilus*) over 48 hours at 30-minute intervals, or at 60-minute intervals for 72 hours under anaerobic conditions for *C. butyricum*, *C. sporogenes*, *L. lactis* subsp. *lactis*, and *Lbc. helveticus*. Bacterial growth was measured using a Biosan RTS-1C spectrophotometer (BioSan, Riga, Latvia) at 850 nm (OD_{850}). Samples were thoroughly shaken before each measurement. The impact of polyphosphate mixture - Cremosal AB4 on the growth dynamics of the tested bacteria was analysed based on the growth curves derived from the OD readings.

RESULTS AND DISCUSSION

The effect of the polyphosphate mixture on the growth of nine selected microorganisms commonly found in processed cheeses was evaluated using the spectrophotometric method to measure bacterial growth at 850 nm. The optical cell density assay is a suitable method for rapidly determining the inhibitory effect of a specific substance (**Lee *et al*., 2002**). In our case, the minimal inhibitory concentration was defined as the concentration of phosphate required to cause a decrease in bacterial optical density below 0.075. (the OD value determined after adding the highest concentration of the polyphosphate mixture tested to the culture medium), which corresponds to the method used by **Loessner *et al*. (1997**).

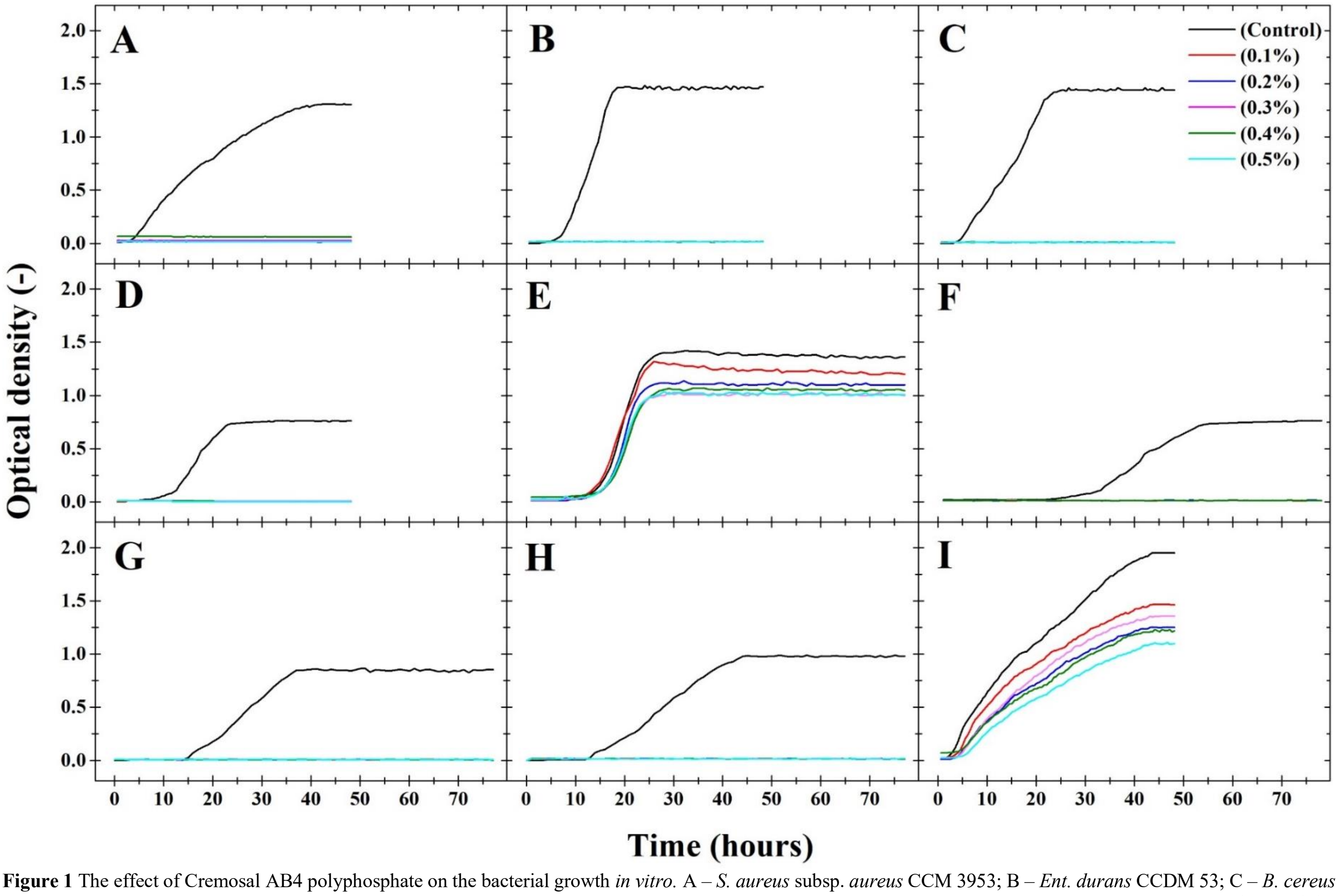

Figure 1 The effect of Cremosal AB4 polyphosphate on the bacterial growth *in vitro.* A – *S. aureus* subsp. *aureus* CCM 3953; B – *Ent. durans* CCDM 53; C – *B. cereus* CCM 2010; D – *G. stearothermophilus* CCM 2062; E – *L. lactis* subsp. *lactis* CCDM 141; F – *Lbc. helveticus* CCDM 142; G – *C. butyricum* CAPM 6342; H – *C. sporogenes* CAPM 6329; I - *Escherichia col*i CCM 3954

The inhibitory effect of Cremosal AB4 polyphosphate mixture on the bacteria tested is shown in Figure 1. At a concentration of 0.1% w/v, Cremosal AB4 polyphosphate mixture was capable to inhibit the growth of all gram-positive bacteria tested except for the *Lactococcus lactis* subsp. *lactis* CCDM 141. No growth inhibition was observed in this strain at any of the applied concentrations of the polyphosphate mixture applied (Fig. 1E). Another study reached a similar conclusion, finding that commercial polyphosphate salt HBS at a concentration of 0.3% w/v is capable of inhibiting the growth of most Gram-positive bacteria tested under experimental conditions (**Loesner *et al.,* 1997).** Maier et al. (1999) studied the impact of HBS on the structure of *B. cereus* and found that HBS concentrations of 0.1% w/v and higher inhibited this bacterium by lysing its cells in the logarithmic phase of growth. **Jen and Shelef, (1986)** and **Lee *et al*., (1994a)** studied the influence of phosphates on *S. aureus* and concluded that the inhibitory concentrations of phosphates ranged from 0.1 to 0.5% w/v, according to the strain of bacteria tested. Several studies have been published focusing on the inhibitory effects of phosphates with varying degrees of condensation on microorganisms (**Buňková *et al*., 2008; Lee *et al*., 1994a, 1994b; Loessner *et al*., 1997; Lorencová *et al*., 2012; Maier *et al*., 1999**). These authors concluded that the higher degree of condensation of polyphosphates in emulsifying salts, the greater their inhibitory effect.

As in the case of *L. lactis* subsp. *lactis* CCDM 141, the polyphosphate mixture did not inhibit the growth of the gram-negative bacterium *Escherichia coli* CCM 3954, which was expected, as it is known from the literature that the antibacterial effect of polyphosphates on gram-negative bacteria is insignificant. These results are consistent with other works (**Knabel *et al.*, 1991; Loessner *et al.*, 1997**), whose results showed no inhibitory effect of emulsifying salts with polyphosphates on the gram-negative bacteria studied. Similarly, **Vareltzis *et al.* (1997)** found no significant inhibitory effect of sodium polyphosphate salt on gram-negative bacteria contaminating the poultry surface.

In laboratory conditions, the values of the minimum inhibitory concentration of Cremosal AB4 polyphosphate mixture are low. Nevertheless, it can be assumed that in complex matrices such as foods (e.g., meat products or processed cheeses), it will be necessary to increase the minimum concentration with an inhibitory effect on the microorganisms present, particularly gram-positive bacteria, including spore-forming bacteria. This assumption is supported by the findings of **Loessner *et al.* (1997)** and **Varga (2005)**, who recommend a polyphosphate concentration of at least 0.5% to slow down the growth of unwanted microbiota in processed cheeses. The need to use higher concentrations of polyphosphates to inhibit microorganisms in real food compared to laboratory conditions was also noted by **Rajkowski *et al.* (1994)** for *Staphylococcus aureus* and *Listeria monocytogenes* in UHT milk and **Lee *et al.* (1994a)** for *S. aureus* in minced meat.

CONCLUSION

The *in vitro* conditions revealed that Cremosal AB4 polyphosphate mixture has an antibacterial effect on the growth behaviour of selected gram-positive bacteria of the genera of *Staphylococcus, Enterococcus, Lactobacillus, Bacillus, Geobacillus* and *Clostridium,* which are significant in the food industry. The minimum inhibitory concentration of this polyphosphate mixture, which stopped the growth of the above-mentioned bacteria, was 0.1% (w/v). The addition of Cremosal AB4 polyphosphate mixture to foodstuffs can thus help to increase their shelf life.c.

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