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Research Note

Inactivation of Calcium-Dependent Lactic Acid Bacteria Phages by Phosphates

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ABSTRACT

The capacity of three phosphates to interrupt the lytic cycle of four specific autochthonal bacteriophages of lactic acid bacteria used as starters was assayed. The phosphates used (polyphosphates A and B and sodium tripolyphosphate–high solubility [TAS]) were selected on the basis of their capacity to sequester divalent cations, which are involved in the lytic cycle of certain bacteriophages. The assays were performed in culture media (deMan Rogosa Sharpe and Elliker broths) and reconstituted (10%, wt/vol) commercial skim milk to which phosphates had been added at concentrations of 0.1, 0.3, and 0.5% (wt/vol). Phosphate TAS was the most inhibitory one, since it was able to inhibit the lytic cycle of all bacteriophages studied, in both broths and milk. In broth, polyphosphates A and B inhibited the lytic cycle of only two bacteriophages at the maximal concentration used (0.5%), whereas in milk, they were not capable of maintaining the same inhibitory effect.

Phosphates are considered GRAS (generally recognized as safe) compounds. They are widely used as food additives, especially in the meat and dairy industries, because of their functional properties. Besides, antimicrobial effects have been reported for gram-positive bacteria that are, in general, more sensitive than gram-negative bacteria *(21, 27, 32, 38, 39).* Their functional properties are related to the ability to enhance flavor, increase yields (due to water retention), and emulsify oils *(26),* while their antibacterial ability has been linked to the capacity for complexing essential metal ions for cell growth *(12, 14, 16–18).* Their inhibitory activity on molds has scarcely been documented *(26, 40).*

Bacteriophage infections of lactic acid bacteria (LAB) represent one of the most persistent and stubborn problems in the dairy industry; thus, considerable efforts have been aimed at minimizing this problem. Several control strategies, such as the use of sanitizers, thermal and chemical treatments *(2, 4, 29, 31, 41),* strain rotation regimes *(24, 25, 33),* and spontaneous mutant phage-resistant strains *(8, 30),* must be used in dairy industries. The use of phage-inhibitory media (PIM) that contain chelating agents (phosphates and citrates) in their composition can control bacteriophages, which require Ca^{2+} to complete their lytic cycle *(44).* Chelating agents are able to inhibit bacteriophage proliferation by binding divalent cations *(6, 9, 36, 37),* interfering with phage adsorption *(3, 22, 23),* or inactivating phage particles *(1, 11, 43, 45).* The direct addition of phosphates to milk destined for dairy fermentations could be an important strategy for inhibiting phages of LAB.

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The aim of this study was to obtain data about the inactivation of autochthonal calcium-dependent phages of LAB by food-grade phosphates.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and culture conditions. The host strains and bacteriophages used in this study are listed in Table 1. The identity confirmation of autochthonal bacteriophages was made by molecular techniques. *Lactococcus lactis* phages were classified by the use of the multiplex PCR method *(15),* and host strains of *Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus paracasei* phages were identified by the use of PCR-ARDRA (amplification and restriction analysis of the 16S rRNA gene) *(35)* and species-specific PCR *(7),* respectively. All phages were isolated in our laboratory from abnormal fermented dairy product manufacturing of Argentinian plants, while host strains were obtained from the commercial starters used in these processes.

Strains were maintained as frozen stocks at −80°C in reconstituted (10%, wt/vol) skim milk and deMan Rogosa Sharpe (MRS) broth (lactobacilli) or Elliker broth (cocci) (Biokar, Beauvois, France) supplemented with 15% (vol/vol) of glycerol and routinely reactivated overnight at optimal growth temperature in the corresponding medium. Phage stocks were prepared as described by Neviani et al. *(28)* in MRS or Elliker broth, added to 10 mM $CaCl_2$ (MRS-Ca/Elliker-Ca), stored at 4°C, and then frozen at −80°C in the presence of 15% (vol/vol) of glycerol.

Phage enumerations (PFU per milliliter) were performed by the double-layer plate titration method *(42),* by using MRS or Elliker agar added to 10 mM $CaCl_2$ and 100 mM glycine *(20).*

Phosphates. Three available food-grade phosphates (SUDAMFOS S.A., Buenos Aires, Argentina) were used: polyphosphates A and B (15 to 20 phosphate units) and sodium tripolyphosphate–high solubility (TAS). These phosphates were selected

TABLE 1. *Origin of phages and their sensitive strains*

Strain	Phage	Phage source
C12[a]	QF9	Fymbo cheese whey
13-3[a]	046	Dambo cheese whey
Ib3[b]	Ib3	Yogurt
A[c]	MLC-A	Probiotic fermented milk

[a] *L. lactis.*
[b] *L. delbrueckii* subsp. *lactis.*
[c] *L. paracasei.*

because of their high ability to sequester divalent cations (Table 2), according to the information provided by the suppliers.

Sensitivity of host strains to phosphates. This assay was carried out to determine if phosphates added at different concentrations (0.1, 0.3, and 0.5%, wt/vol) were capable of inhibiting the growth of lactic acid strains. For this aim, MRS (lactobacilli) and Elliker (cocci) broth were used as basal media. Stock solutions (5%, wt/vol) of phosphates were prepared in distilled water and subsequently sterilized by filtration (0.45-μm-pore-size membranes, Millipore, Bedford, Mass.) *(17).* MRS-phosphate or Elliker-phosphate media were prepared with distilled water and the necessary volumes of neutralized (until pH = 7.0 ± 0.2) phosphate stock solutions to reach the final phosphate concentrations cited. Strains were inoculated (1%, vol/vol) in tubes with 5 ml of the phosphate broths and incubated for 24 h at their optimal growth temperature (30°C for *L. lactis,* 37°C for *L. paracasei,* and 42°C for *L. delbrueckii* subsp. *lactis*). Tubes of broth without phosphates were used as controls. An A_{600} value (as growth measurement) that was lower than 0.2 was considered inhibition *(21).* The assays were performed three times.

Inhibition of bacteriophage lysis. MRS (lactobacilli) and Elliker (cocci) broths with added phosphates (0.1, 0.3, and 0.5%, wt/vol) and $CaCl_2$ (10 mM) were used. Tubes were inoculated (1%, vol/vol) with host strain overnight cultures and the corresponding bacteriophages (multiplicity of infection ≅ 1) and incubated for 8 h at optimal temperatures (until good growth was observed in control tubes, without phosphates). Results were registered as + (lysis, optical density at 600 nm [OD_{600}] < 0.2), ± (partial lysis, OD_{600} between 0.2 and 0.8), and − (absence of lysis, OD_{600} > 1.0). The assays were performed three times.

Milk acidification kinetics. These assays were performed in reconstituted (10%, wt/vol) commercial skim milk with added phosphates (0.1, 0.3, and 0.5%, wt/vol). Tubes were inoculated with strains and bacteriophages, as described previously, and incubated at strain growth optimal temperatures. At intervals, pH values were determined, and acidification kinetics were obtained. Reconstituted skim milk without phosphates was used as a control. The assays were performed three times.

TABLE 2. *Sequestering power of phosphates used in this study (data provided by the supplier)*[a]

Phosphate	pH (±0.2)	
	7.0	Natural[b]
A	15	15[c]
B	15	15[d]
TAS	12	13[e]

[a] Values for 1.2% (wt/vol) phosphate expressed as grams of Ca per 100 g of phosphate.
[b] pH value corresponding to a phosphate solution at 1.2%.
[c] pH = 6.8.
[d] pH = 6.5.
[e] pH = 9.6.

TABLE 3. *Inhibition of the lytic cycle of lactic acid bacteria phages (Elliker broth-lactococci and MRS broth-lactobacilli) by the addition of phosphates*[a]

Phage	Strain	Lysis control		Phosphate (% wt/vol)								
				A			B			TAS		
		With Ca[b]	Without Ca	0.1	0.3	0.5	0.1	0.3	0.5	0.1	0.3	0.5
QF9	C12[c]	+	−	+	+	+	+	+	+	+	±	−
046	13-3[c]	+	−	+	+	−	+	+	−	+	−	−
Ib3	Ib3[d]	+	−	+	+	+	+	+	+	+	−	−
MLC-A	A[e]	+	−	+	+	−	+	+	−	+	±	−

[a] +, lysis (OD_{600} < 0.2 after 8 h); ±, partial lysis (OD_{600} value between 0.2 and 0.8 after 8 h); −, absence of lysis (OD_{600} > 1.0 after 8 h).
[b] Ca^{2+} final concentration = 10 mM.
[c] *L. lactis.*
[d] *L. delbrueckii* subsp. *lactis.*
[e] *L. paracasei.*

RESULTS

Sensitivity of host strains to phosphates. The four strains studied were able to grow (OD_{600} > 1.0 after 24 h) at all phosphate concentrations assayed (data not shown).

Bacteriophage lysis inhibition. Phosphate TAS was more inhibitory than polyphosphates A and B, since it was able to inhibit the lytic cycle of all bacteriophages assayed at concentrations that were dependent on the strain-phage system studied (Table 3). For phages 046 and MLC-A, polyphosphates A and B were capable of inhibiting cell lysis only when the maximal concentration was used (0.5%).

Milk acidification kinetics. When reconstituted skim milk (10%, wt/vol) was used to perform the assays, the results were different from those obtained in broth. Phosphate TAS at 0.3% was not able to stop the cell lysis produced by bacteriophages QF9 and MLC-A (data not shown), as was observed partially in broth (Table 3). For these phages, a concentration of 0.5% was necessary to inhibit phage infections in milk (Figs. 1 and 2). At 0.5%, phosphates A and B did not inhibit the lytic cycle of bacteriophages 046 and MLC-A (data not shown). However, this concentration had been sufficient to produce the inhibition in broth (Table 3). On the other hand, it was possible to observe an inhibition of cell lysis for phage 046 (*L. lactis*) and phage Ib3 (*L. delbrueckii* subsp. *lactis*) in milk (Figs. 3 and 4) because of the addition of 0.3% phosphate TAS, as for that obtained in broth.

DISCUSSION

The antimicrobial activity of phosphates has been reported quite extensively *(27, 39, 47).* However, information

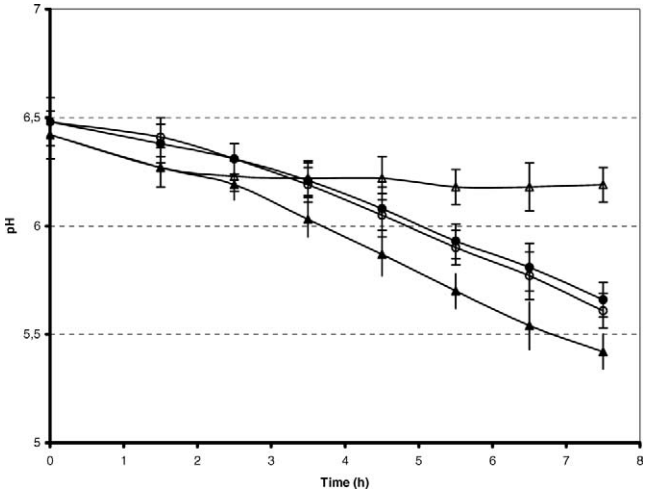

FIGURE 1. *Effect of phosphate addition on the milk acidification kinetics of* L. lactis *Cl2 in the presence of its lytic phage QF9.* ▲, *Cl2;* △, *Cl2/QF9;* ●, *Cl2/phosphate TAS (0.5%);* ○, *Cl2/QF9/phosphate TAS (0.5%). Values are the media of three determinations. Bars represent standard deviation.*

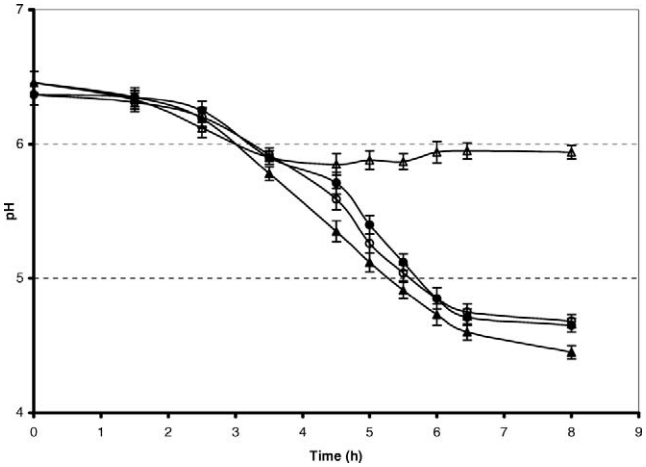

FIGURE 3. *Effect of phosphate addition on the milk acidification kinetics of* L. lactis *13-3 in the presence of its lytic phage 046.* ▲, *13-3;* △, *13-3/046;* ●, *13-3/phosphate TAS (0.3%);* ○, *13-3/046/phosphate TAS (0.3%). Values are the media of three determinations. Bars represent standard deviation.*

about their use as food preservatives is scarce as yet. Their inhibitory activity has been linked with their capacity to complex divalent cations (Ca^{2+}, Mg^{2+}, and Fe^{2+}), essentials for cell life *(12, 14, 17, 46).* This mechanism was also suggested for mold inhibition, since the phosphates with a higher cation sequestering capacity were the most inhibitory *(14, 40).* The direct addition of phosphates in milk could be a simple methodology to help protect LAB starter bacteria against Ca^{2+}-dependent bacteriophages. Concentrations of phosphates between 1.6 and 2.0% in milk produced physicochemical instability in casein and decreased cheese yield *(10).* Casein in milk acts as a multivalent ion that can undergo ion-exchange reactions. This leads to a reduction in the levels of free calcium ions and an increase in bound calcium or ''colloidal calcium'' as phosphates are added *(26).* With certain concentrations of added phosphates, casein forms with them a stable, large complex. However, if an excess is added (above that required for formation of this complex), the caseinate particles release their attached colloidal inorganic salts, which results in the complete dispersion of the casein micelles, with a resultant loss in the normal opacity of the milk. The phosphate concentrations used by us were lower (0.5% as maximum) than those used in PIM formulations *(44),* and negative effects on milk casein stability were not observed.

Previous studies *(10)* about the design of media with phosphates to inhibit phages were focused mainly on mesophilic LAB (*Lactococcus* genus) and their phages. However, the use of thermophilic LAB as starters in cheese and fermented milk production is worldwide. In addition, *L. paracasei* is nowadays one of the most widely used species in fermented dairy products as a probiotic microorganism *(19).* Our results demonstrated that the LAB strains used

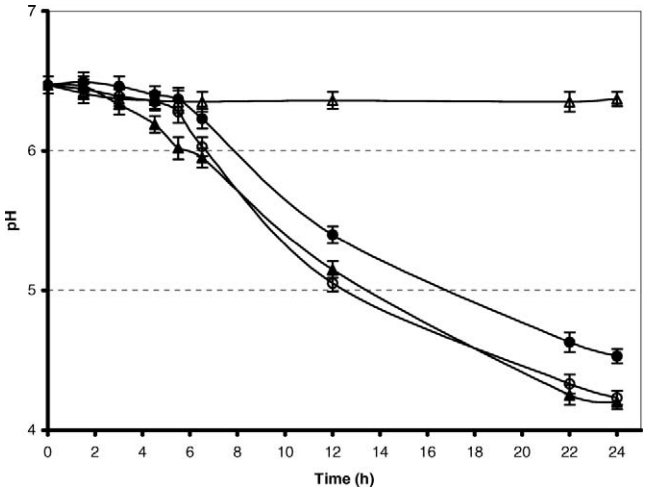

FIGURE 2. *Effect of phosphate addition on the milk acidification kinetics of* L. paracasei *A in the presence of its lytic phage MLC-A.* ▲, *A;* △, *A/MLC-A;* ●, *A/phosphate TAS (0.5%);* ○, *A/MLC-A/phosphate TAS (0.5%). Values are the media of three determinations. Bars represent standard deviation.*

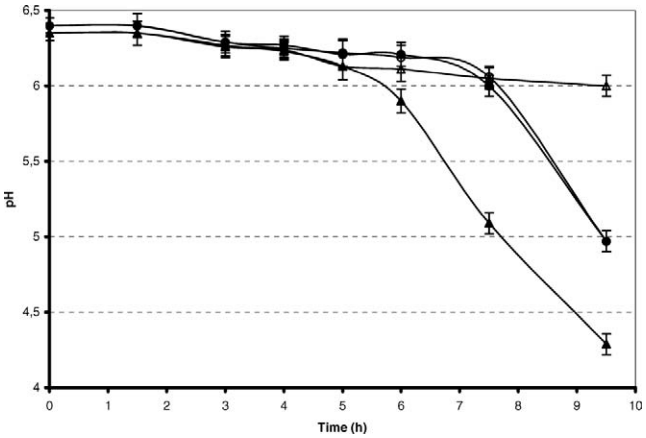

FIGURE 4. *Effect of phosphate addition on the milk acidification kinetics of* L. delbrueckii *subsp.* lactis *Ib3 in the presence of its lytic phage Ib3.* ▲, *Ib3;* △, *Ib3/Ib3;* ●, *Ib3/phosphate TAS (0.3%);* ○, *Ib3/Ib3/phosphate TAS (0.3%). Values are the media of three determinations. Bars represent standard deviation.*

(*L. lactis* Cl2, *L. lactis* 13-3, *L. delbrueckii* subsp. *lactis* Ib3, and *L. paracasei* A) were able to grow at 0.5% concentrations of phosphates.

The calcium ion requirement for the proliferation of bacteriophages has been observed in several phage-cell systems. The bacteriophages used in our study required calcium after adsorption, since adsorption rates (at 30 min), with and without phosphates, were, in all cases, higher than 96% (data not shown). Reiter *(34)* reported the inhibition of lactic starter bacteriophages in a calcium-deficient medium. The inhibition of phage replication was also confirmed in starters grown in milk treated with phosphate salts to bind the calcium *(44)*. These findings led to the development of PIM, most of which were prepared from deionized whey supplemented with hydrolyzed proteins and one or more cation-scavenging compounds (e.g., phosphates, citrates) to bind calcium. In spite of serving a useful function in protecting strains from phage attacks, PIM has been shown by some authors *(5, 44)* to decrease the ability to support good growth of LAB cultures. Previous studies *(13, 48)* have shown that the high concentrations (1.6 to 2%) of phosphates used in PIM were inhibitory to LAB cell growth. The results obtained by us demonstrated, as preliminary evidence, that TAS added to milk at low concentrations (0.3 to 0.5%) was effective at inhibiting the lytic cycle of specific bacteriophages of thermophilic and mesophilic LAB in milk. However, some strains (such as *L. delbrueckii* subsp. *bulgaricus* Ib3) could show a delay in their acidifying activity, probably due to the buffer effect produced by phosphates added to milk.

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