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Optimization of Methodology to Enumerate *Lactobacillus delbrueckii* Phages

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ABSTRACT

The influence of incubation temperature, presence of calcium cations in soft-agar (double-layer plaque titration), nature of inoculum used (from broth or reconstituted skim milk, RSM) and addition of glycine on enumeration of *Lactobacillus delbrueckii* phages was studied. Assays were performed on two temperate and three virulent *Lactobacillus delbrueckii* phages. Results showed that the diverse conditions influenced the number and definition of phage plaques. The addition of calcium to the soft-agar increased (ANOVA test, $P < 0.05$) one log order the counts of all phages studied. The presence of glycine improved the definition and size of plaques for some phages, but not their counts. The origin of inoculum was important for phage Cb1/204, since plaques were more visible when an inoculum from RSM was used.

Some species of lactic acid bacteria phages are fastidious for counting, and an optimized methodology can allow overcoming this problem. This study demonstrates enhanced detection of phage particles, assuring the correctness of visualization and quantification of them.

INTRODUCTION

Bacteriophage infections are known to be one of the main causes of loss of starter acidifying activity at cheese and fermented milk factories *(7, 8)*, leading to serious technological problems *(1, 2, 4)*.

Economic losses due to phage infections make it necessary to detect and minimize their presence in both lab and industrial environments to reduce the attacks and obtain normal fermentations. To achieve this, an optimized methodology that allows precise enumeration and detection of lysis plaques is necessary.

The conventional method used to enumerate active phage particles is double-layer plaque titration *(17)*. Several factors can influence the size and definition of plaques and affect phage counts. Although some phages need divalent cations (such as Ca^{2+} and Mg^{2+}) to complete the lytic cycle *(11, 13, 14, 16, 18)*, most are able to infect bacterial cells in the absence of these ions *(3, 10, 11, 14)*. In order to obtain the best results with the methodology used, it is fundamental that calcium is available for those systems that need it to complete the lytic cycle. Another important factor reported to obtain visible plaques is the presence of glycine *(6)* in the culture medium. However, the importance of glycine has not been demonstrated completely for *Lacto-*

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TABLE 1. Phages and their host strains used in this study

Phage	Phage type	Host strain
Cb1/204[a]	Temperate	*L. delbrueckii* subsp. *lactis* 204[c]
Cb1/342[a]	Temperate	*L. delbrueckii* subsp. *bulgaricus*342[c]
BYM[b]	Virulent	*L. delbrueckii* subsp. *lactis* YSD V[d]
YAB[b]	Virulent	*L. delbrueckii* subsp. *lactis* Ab1[d]
Ib3[b]	Virulent	*L. delbrueckii* subsp. *bulgaricus* Ib3[d]

[a]isolated from commercial strain *L. delbrueckii* subsp. *lactis* Cb1

[b]isolated from a failed manufacture of yogurt

[c]wild strain isolated from natural whey starters

[d]commercial strain

FIGURE 1. Lysis plaques obtained for phage Cb1/204, using an inoculum of its host strain (*Lactobacillus delbrueckii* subsp. *lactis* 204) obtained from RSM diluted in MRS broth (DO_{560nm} = 0.70) and from MRS overnight diluted (DO_{560nm} = 1) and incubated at 34°C (A, B), 37°C (C, D) and 42°C (E, F)

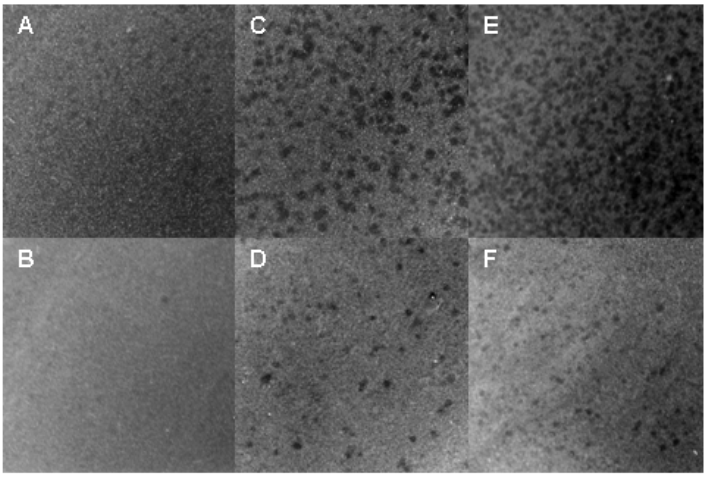
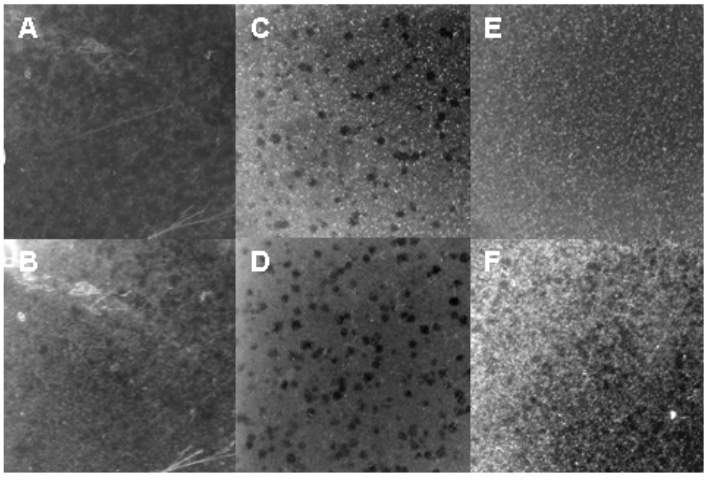


FIGURE 2. Lysis plaques obtained for phage Cb1/342, using an inoculum of its host strain (*Lactobacillus delbrueckii* subsp. *bulgaricus* 342) obtained from RSM diluted in MRS broth (OD_{560nm} = 0.70) and from MRS overnight diluted (DO_{560nm} = 1) and incubated at 34°C (A, B), 37°C (C, D) and 42°C (E, F)



bacillus delbrueckii bacteriophages, either virulent or temperate ones. In addition, growth temperature can influence the characteristics of the plaques, especially if the burst size is low. For this reason, it could be appropriate to incubate the host cells at suboptimal temperatures, providing each phage-strain system the most favorable conditions for plaque enumeration.

The aim of this study was to establish the best conditions for enumeration of *Lactobacillus delbrueckii* phages in order to optimize their counts.

MATERIALS AND METHODS

Bacterial strains and bacteriophages

Phages and their host strains used in this study are shown in Table 1. They were all isolated at INLAIN (Instituto de Lactología Industrial, Santa Fe, Argentina) from defective industrial processes of fermented milks (virulent phages Ib3, YAB and BYM) or by induction with mitomycin C (temperate phages Cb1/204 and Cb1/342) *(16)*. *Lactobacillus delbrueckii* strains were grown and routinely reactivated overnight (42°C) in deMan Rogosa Sharpe (MRS) broth (Biokar, Beauvois, France). They were maintained as frozen (-80°C) stocks in sterile reconstituted (10% wt/vol) commercial nonfat dry skim milk (RSM). Phage stocks were prepared as described by Neviani et al. *(9)* and stored at 4°C (MRS broth) and -80°C (MRS broth with 15% vol/vol of glycerol).

TABLE 2. Statistical analysis (one-way ANOVA) of calcium availability influence on bacteriophage counts ($P < 005$)

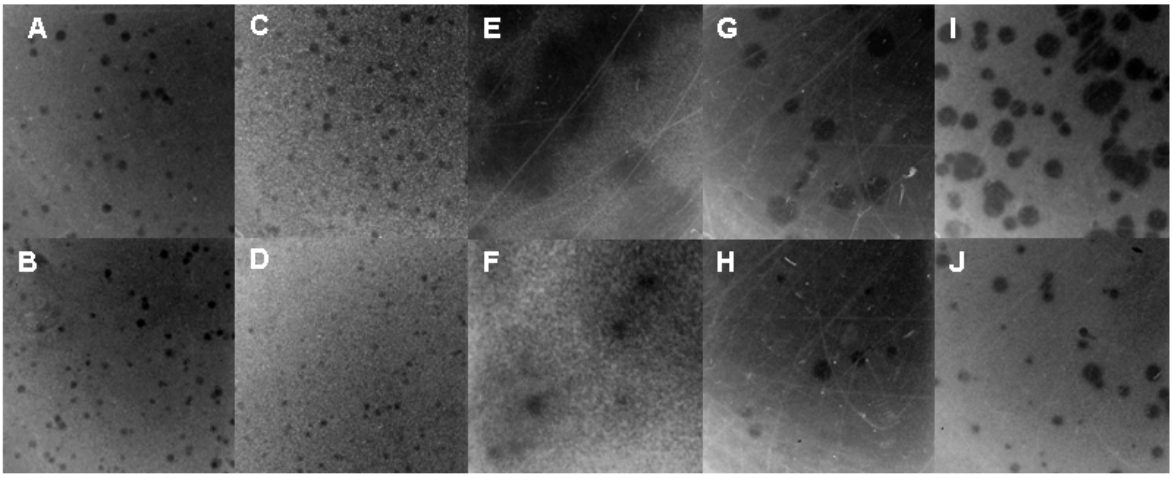
	Probability level of statistic treatment (P)[c] between counts with addition of calcium in:		
Phage	**Agar and soft-agar layers vs. agar layer**	**Agar and soft-agar layers vs. soft-agar layer**	**Agar layer vs. soft-agar layer**
Cb1/204[a]	0.0264	0.6565	0.0007
Cb1/342[a]	0.0198	0.8338	0.0337
BYM[b]	0.0263	0.6701	0.0001
YAB[b]	0.0008	0.2637	0.0001
Ib3[b]	0.0054	0.6802	0.0159

[a]Temperate phages isolated from commercial strain *L. delbrueckii* subsp. *lactis* Cb1

[b]Virulent phages isolated from failed yogurt manufactures

[c]$P < 0.05$ = statistically significant difference

FIGURE 3. Lysis plaques obtained with and without addition of glycine (100 mM) to MRS agar for temperate phages Cb1/342 (A, B) and Cb1/204 (C, D) and virulent phages YAB (E, F), Ib3 (G, H) and BYM (I, J)



Inoculum conditions and temperature influence

Host strains of respective phages were inoculated into plates from MRS broth and RSM (10% wt/vol) overnight cultures. The inoculum to make the enumeration from RSM was obtained after a dilution in MRS broth (final OD_{560nm} = 0.70), while the inoculum from MRS was obtained after a dilution in the same medium (final OD_{560nm} = 1.05).

Phage enumerations (plaque forming units per milliliter, PFU) were performed using the double-layer plaque titration method *(17)*, using MRS as culture medium. Culture media were prepared immediately before the assays. Three incubation temperatures (34, 37 and 42°C) were selected. All assays were performed in triplicate. The plaques were counted and the plates photographed to compare the size of lysis plaques.

Glycine and calcium influence

Assays were performed with and without the addition of glycine (final concentration 100 mM) in the MRS bottom layer for phage titrations *(6)*.

The influence of calcium on bacteriophage plaque formation was studied, using the double-layer plate titration method modified as follows: MRS bottom agar (1.2% w/v agar) layers with and without $CaCl_2$ (10 mM) and MRS soft (top; 0.6% w/v agar) agar layers with and without $CaCl_2$ (50 mM) were used. For these two determinations, virulent bacteriophages (Ib3, YAB and BYM) were included in the study. All tests were conducted in triplicate.

Statistical analysis

Statistical analysis was conducted using one-way ANOVA, taking a probability level of $P < 0.05$ to indicate statistical significance. This analysis was applied only to the results of tests of the influence of calcium on phage counts.

FIGURE 4. Lysis plaques obtained for phage Cb1/204, using $CaCl_2$ in both layers (agar and soft agar, 10 mM and 50 mM, respectively) (A), only in the bottom agar layer (B), and only in the soft agar layer (C); these conditions were the same for phage Cb1/342 (D, E and F)

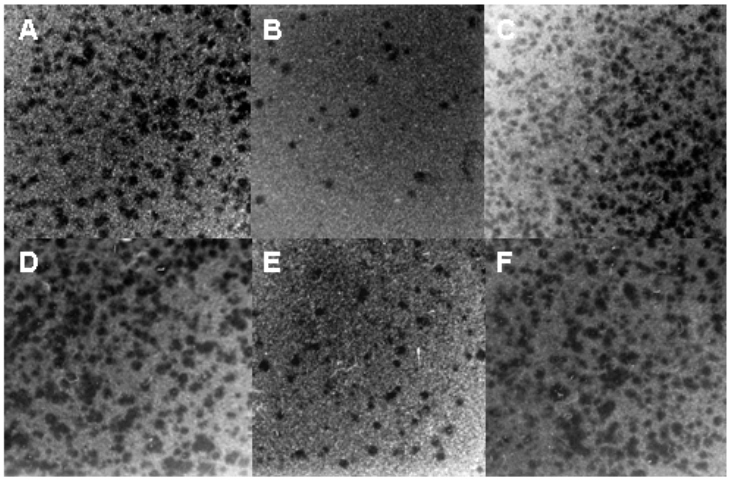
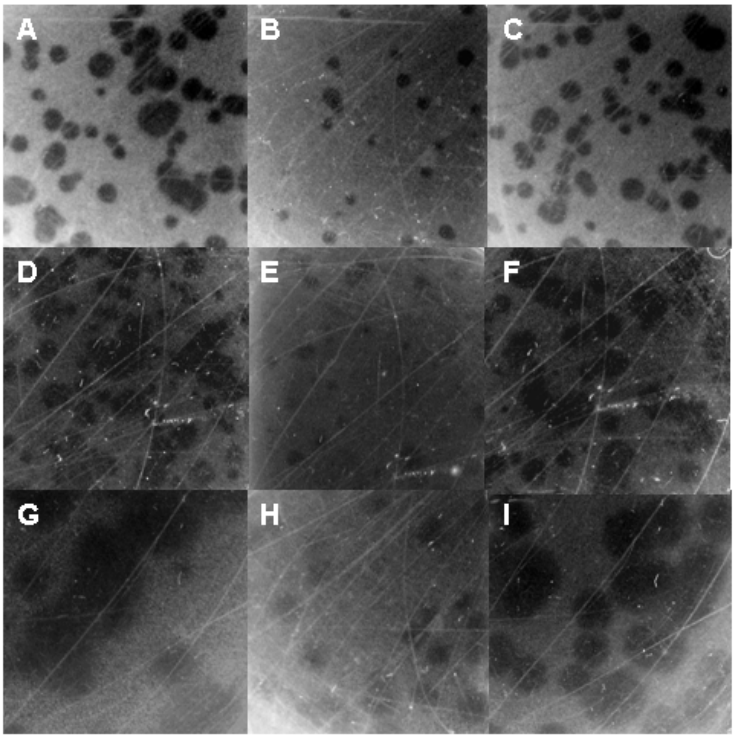


FIGURE 5. Lysis plaques obtained for phage BYM, using $CaCl_2$ in both layers (bottom and soft agar, 10 mM and 50 mM, respectively) (A) only in the bottom agar layer (B), and only in the soft agar layer (C); these conditions were the same for phages Ib3 (D, E and F) and YAB (G, H and I)



RESULTS

The best visualization of plaques was obtained when an inoculum from a milk culture of the host strain (phage Cb1/204) (Fig. 1) and from an MRS broth culture of the host strain (phage Cb1/342) (Fig. 2) were used. The inoculum used for phage titrations did not influence phage Cb1/342 counts. In contrast, phage Cb1/204 counts were lower when MRS cultures were used.

When plates were inoculated at 34°C, temperate phages did not produce lysis plaques. Temperatures of 37 and 42°C were favorable for enumeration of phage Cb1/204 plaques (Fig. 1), and similar counts were obtained at both temperatures. However, phage Cb1/342 revealed clearer plaques at 37°C (Fig. 2), showing slightly higher counts than those obtained at 42°C.

The presence of glycine influenced considerably the definition and size of lysis plaques for virulent phages, but not for temperate phages (Fig. 3). Plaque number was not affected by the presence of glycine, either for virulent or temperate phages (data not shown).

For all phages (temperate and virulent) the addition of calcium in the soft agar layer significantly altered their enumeration. In all cases, phage counts were at least one log order higher when calcium was used in the soft agar, whether or not the MRS agar contained calcium, in comparison to those titrations where calcium was not added to the soft agar layer (Fig. 4 and 5). Statistical analysis confirmed these differences ($P < 0.05$) (Table 2). On the other hand, we demonstrated that the addition of calcium to the bottom layer could be unnecessary, since no significant differences were observed in the counts, compared to the counts when calcium was added only to the soft layer.

DISCUSSION

In Argentina, phage control strategies are mainly used to protect thermophilic dairy starters (*Streptococcus thermophilus* and *L. delbrueckii*), which are widely used in cheese and fermented milk processes *(12, 15)*. The double-layer plaque titration *(17)* is the method used worldwide as the reference for counting active phage particles. However, sometimes this methodology requires optimization, because its efficiency can vary depending upon the bacterium/phage system studied.

Several factors can affect the visualization of phage plaques and thus influence phage counts. A previous study *(6)* reported that the addition of glycine to the bottom layer improved the plaque size of poor plaque-producing temperate lactococcal bacteriophages. However, this variable was not tested for phages of other LAB species. In this work, the addition of glycine was tested on temp-

erate and virulent phages, demonstrating its influence on plaque size and definition in all cases, but mainly on the virulent phages studied (YAB, Ib3 and BYM). No significant effect on enumeration was observed.

A calcium ion requirement for proliferation and plaque formation for LAB bacteriophages has been demonstrated in several phage-cell systems. According to Sechaud et al. *(13)*, Ca^{2+} (or Mg^{2+}) ions not only stabilize the coiled DNA inside the phage capsid and greatly improve the adsorption rate but also regulate the penetration efficiency of phage DNA into bacterial cells. After adsorption (possibly at the DNA injection step), the divalent cations could act as counter-ions during translocation of the phage DNA across the cellular membrane *(5)* or be involved in DNA stabilization following the injection step. The conventional method of double-layer plaque titration uses Ca^{2+} in the support media (agarized). This study demonstrated that calcium addition to soft agar increased the availability of this cation for the bacteriophage. Significant differences in phage particle counts, of at least one log order, were found for all phages assayed. The data obtained for *L. delbrueckii* (temperate and virulent) phages studied in this work indicate the need to modify methodology for phage counting to include calcium.

In addition, the preparation of the inoculum used was important to define the methodology. The strains cultured overnight in broth and RSM were diluted in broth up to a carefully controlled OD_{560nm} value. This methodology implemented in our laboratory allowed us to increase counts of phage Cb1/204 and also to obtain more visible plaques. In contrast, for Cb1/342 phage, the MRS overnight inoculum was more suitable.

On the basis of our results, each phage/host strain system should be considered individually when performing detection and enumeration of bacteriophages. Even if all these modifications are implemented in other laboratories, at present there is no information about changes of the conventional methodology *(17)*.

ACKNOWLEDGMENTS

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