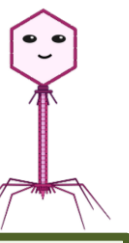




Stability and lytic activity assessment of bacteriophages targeting *Staphylococcus aureus* causing bovine mastitis in milk

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Introduction

Bovine mastitis is a major cause of culling in dairy cattle and the antimicrobial treatment of the infection contributes to the emergence and the spread of antimicrobial resistances. Phage therapy could be a promising approach, but the biological and physicochemical properties of milk can affect the phages properties. The objective of this study was to compare the stability and lytic activity of phages targeting *Staphylococcus aureus* in raw and pasteurized milk.

Material and methods

1. Collection of phages

A total of 28 bacteriophages targeting *S. aureus* were obtained from Kyushu University (Japan). A broad host range was observed for all phages (data not shown).

2. Milk samples

A cow with low somatic cell count (SCC) was milked. Half of the samples was pasteurized at 70°C during 35min. The other half was kept raw. Sterility of the samples was assessed (Figure 1).

3. Stability in milk

The phage inoculum was titrated. Subsequently, milk samples were inoculated and incubated for 6 hours at 37°C before titration (Figure 2). Following this, a one-way ANOVA with Geisser-Greenhouse correction and a Dunnett multiple comparison test were conducted.

4. Lytic activity in milk

A tablet of PBS was added to 100ml of milk to enhance its buffer capacity. The milk samples were inoculated with 250µl of phage (8 log PFU/ml) and/or 250µl of bacteria (5 Log CFU/ml). Subsequently, they were incubated at 37°C and titrated after 6 and 12h incubation (Figure 3). Parametric or non-parametric Two-way ANOVA with Šidák or Bonferonni multiple comparisons were performed.

5. Optimal Multiplicity Of Infection (MOI) in milk

For 9 selected phages, 100 µl of phage at 6 log PFU/ml and 100 µl of host bacteria at a concentration ranging from 3-8 log CFU/ml were inoculated at 37°C in LB broth and titrated after 3,5h of incubation. The MOI that gave the highest phage titer was selected for inoculation of raw milk, pasteurized milk and UHT whole milk (Classic' Lait Entier, Carrefour).

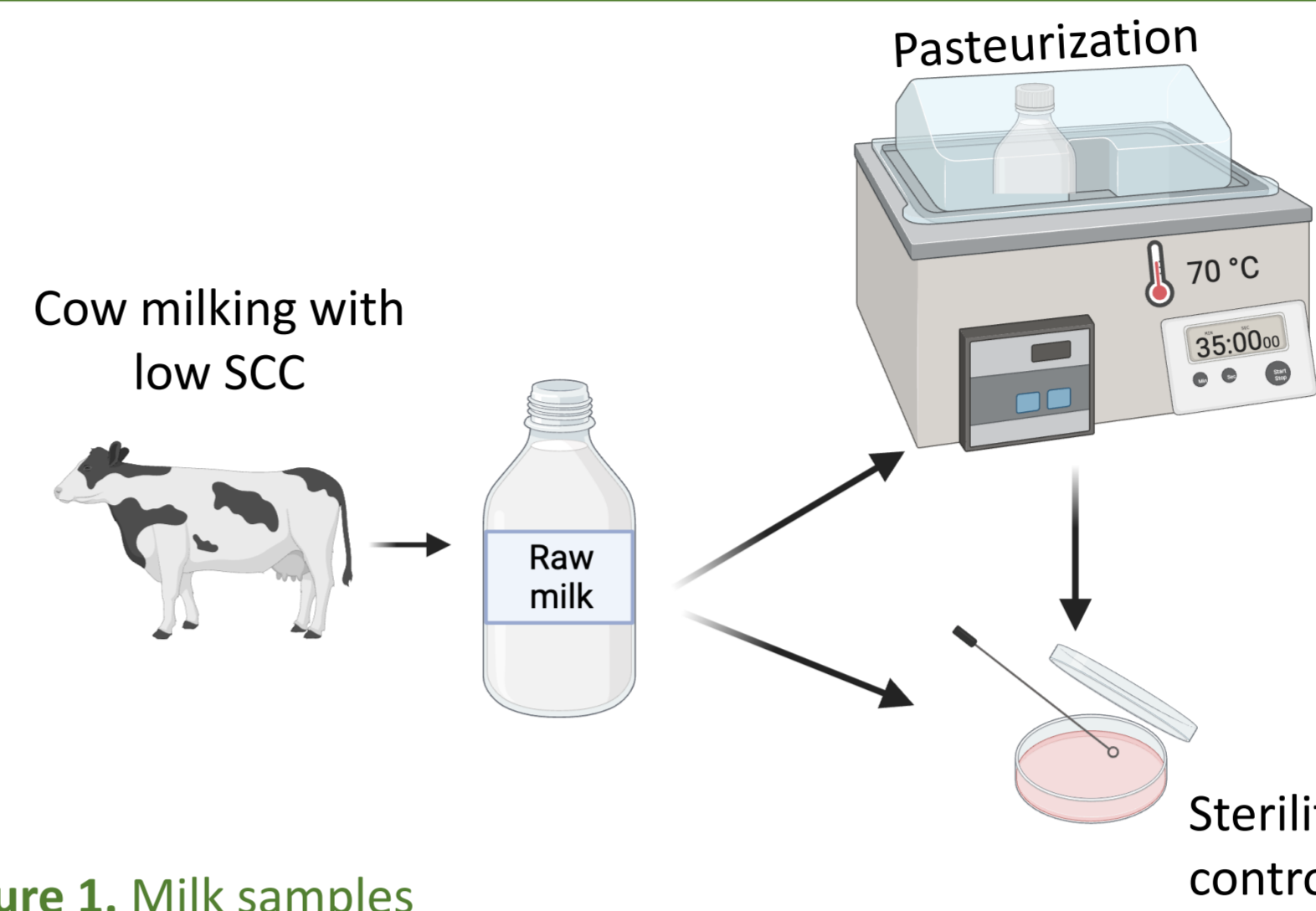


Figure 1. Milk samples

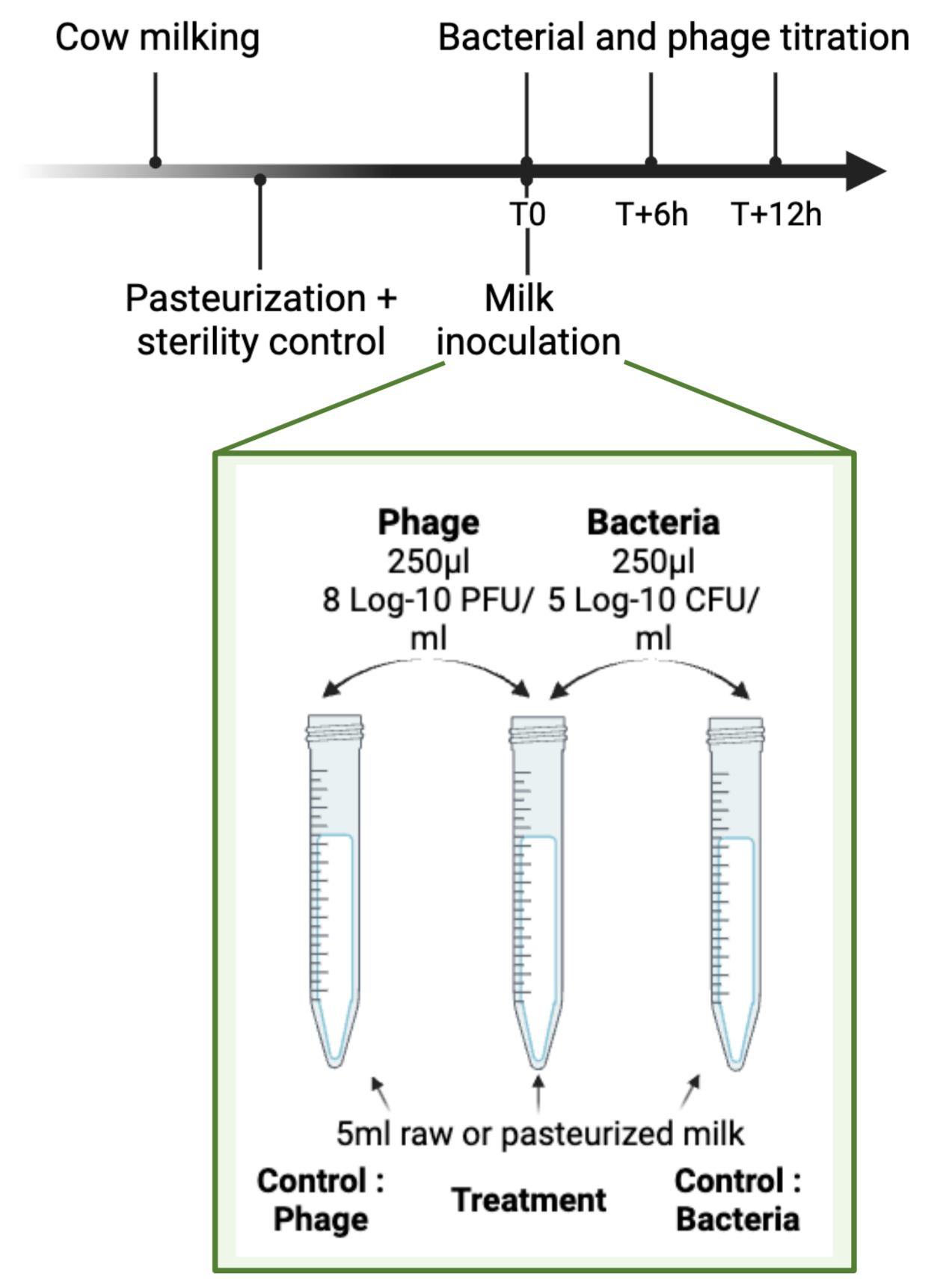


Figure 3. Lytic activity in milk

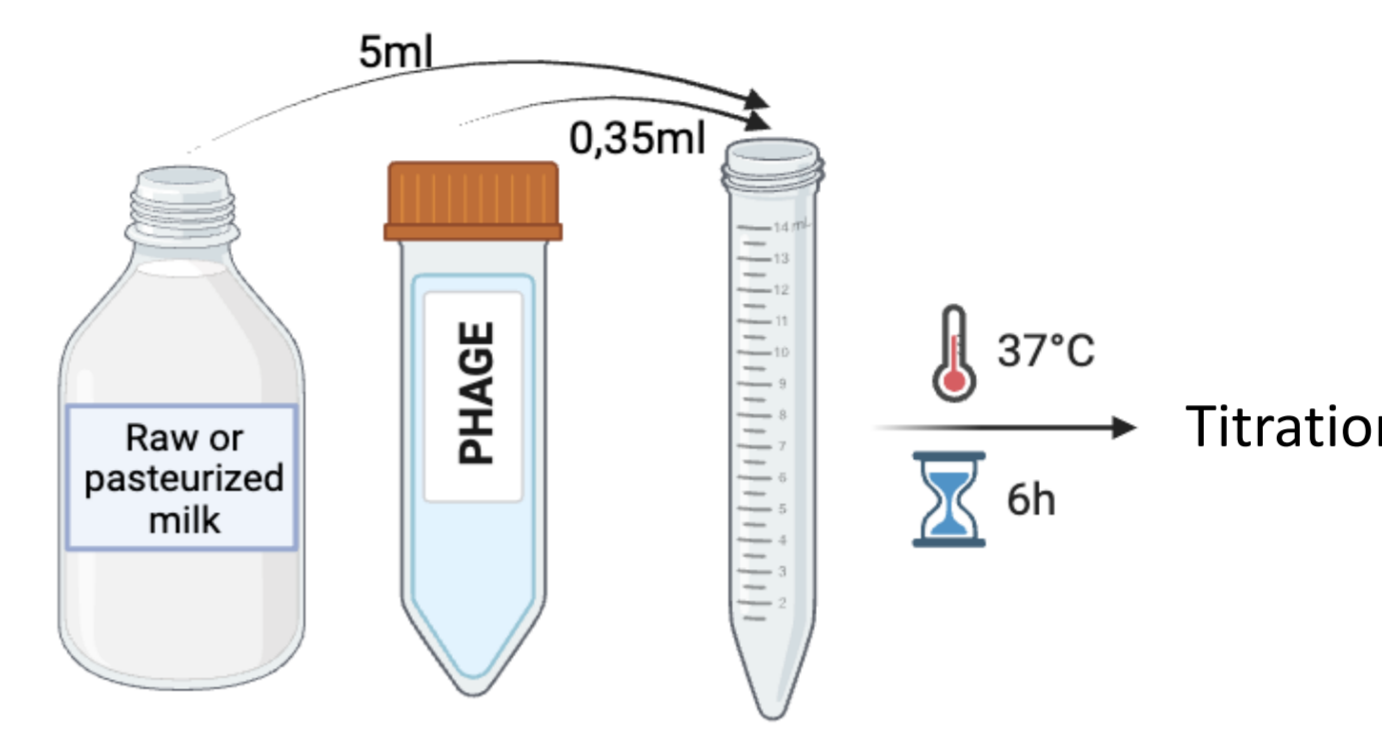


Figure 2. Stability in milk

Results

1. Stability in milk

A significant decrease of phage titer was measured after 6 hours of incubation in milk. The average stability (titer after incubation/titer of inoculum) was 10% in pasteurized milk and 7% in raw milk. A significant difference between phages in pasteurized or raw milk was observed. Multiple comparisons showed the same effect for 5 phages (*) (Figure 5).

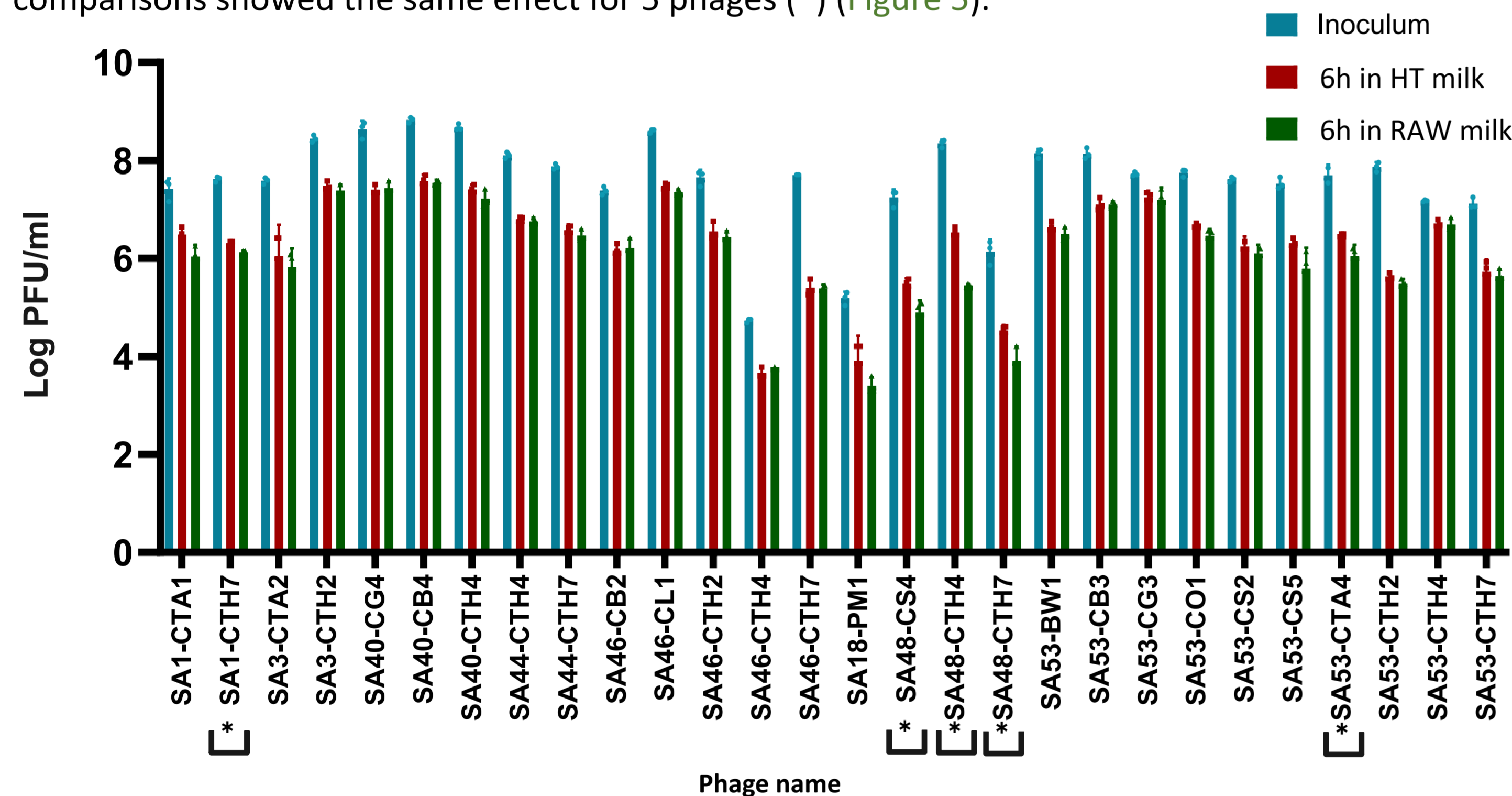


Figure 5. Stability in milk

2. Lytic activity in milk

In the control groups, the phage titer was significantly higher for 9/21 phages in pasteurized milk in comparison with raw milk. No difference was observed for the remaining phages.

In the treatment group, the phage titer increased significantly over time for 11/21 phages in pasteurized milk and 9/21 phages in raw milk. The titer didn't significantly change for 8 phages in both raw and pasteurized milk and significantly decreased for 2/21 phages and 4/21 phages in pasteurized and raw milk. When we compared the pasteurized and raw group, 15/21 phages showed a higher titer in pasteurized groups, the remaining phages didn't show any difference (Figure 6). No decrease in bacterial count was observed.

3. Optimal MOI in milk

Phage SA1-CTA1 demonstrated an optimal MOI of 0,1, while the other phages had their optimal MOI at 0,01. Inoculation of milk with these MOIs showed a reduction in bacterial count of up to 1,2 log CFU/ml in UHT milk. The reduction was highest after 3 hours of incubation. However, in pasteurized and raw milk, phages exhibited minimal to no effect on bacterial titration compared to the control. In some samples and mostly in raw milk, the treated samples (phage + host bacteria) showed a higher bacterial count compared to the control (Figure 7).

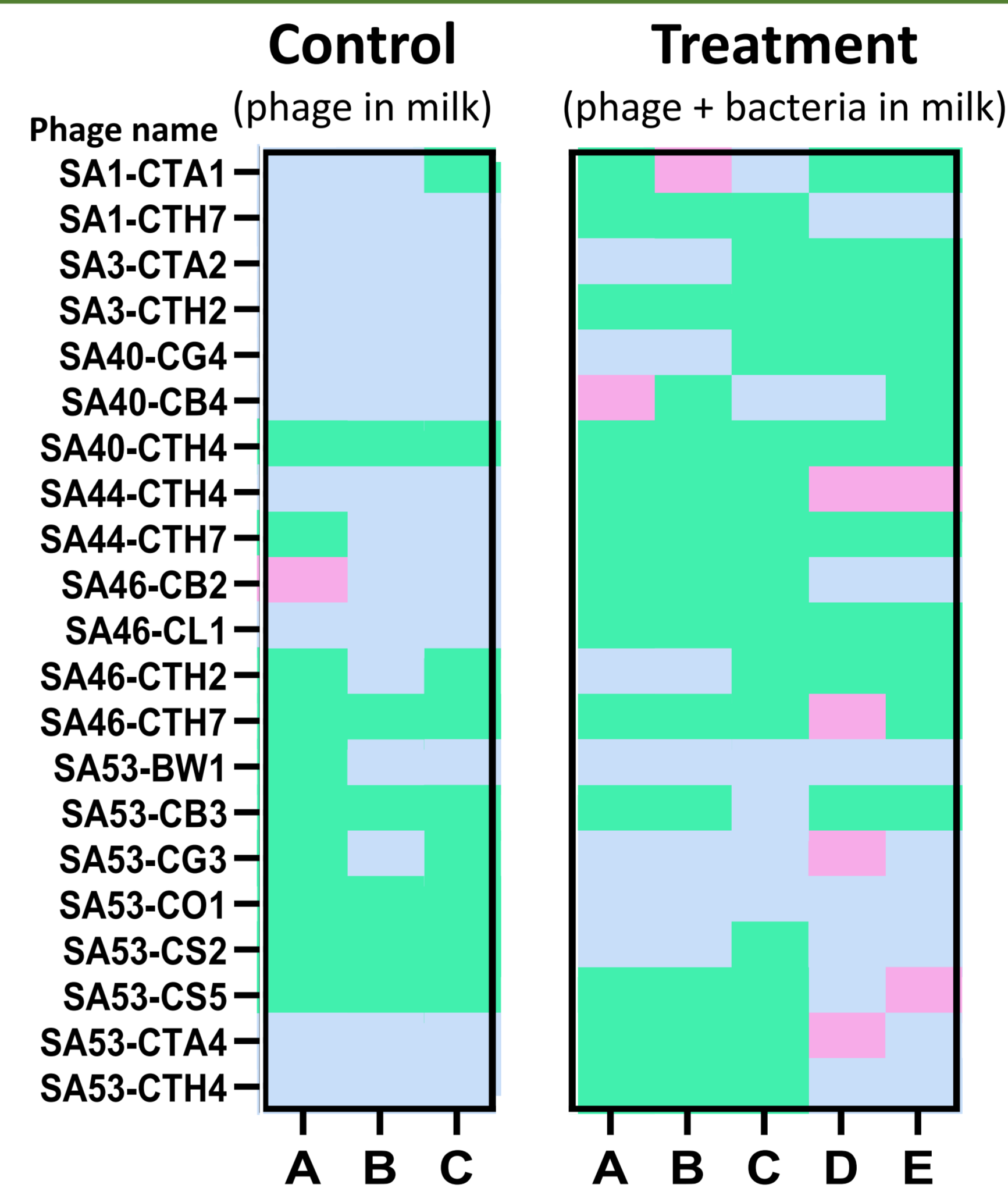


Figure 6. Lytic activity in milk

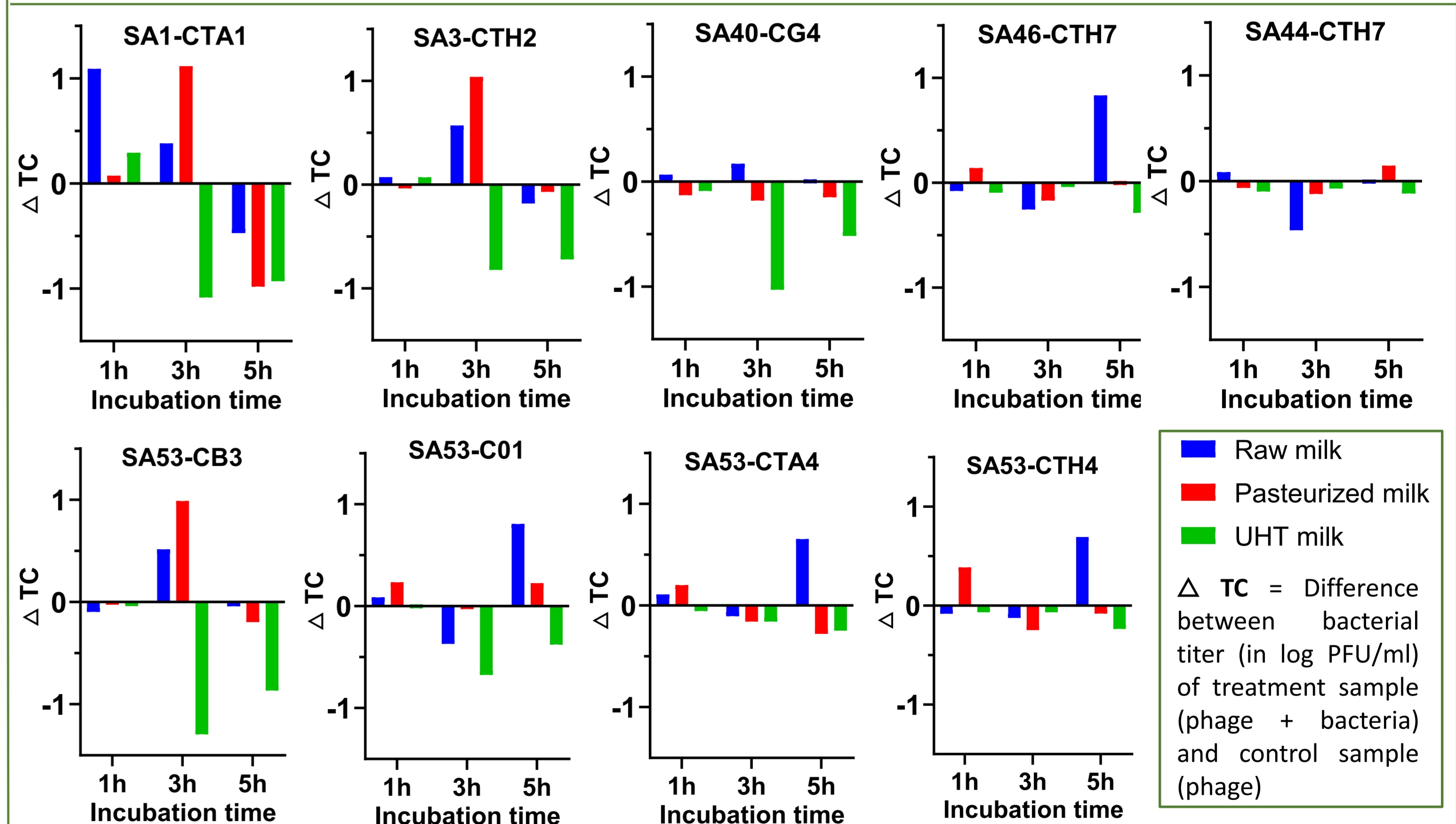


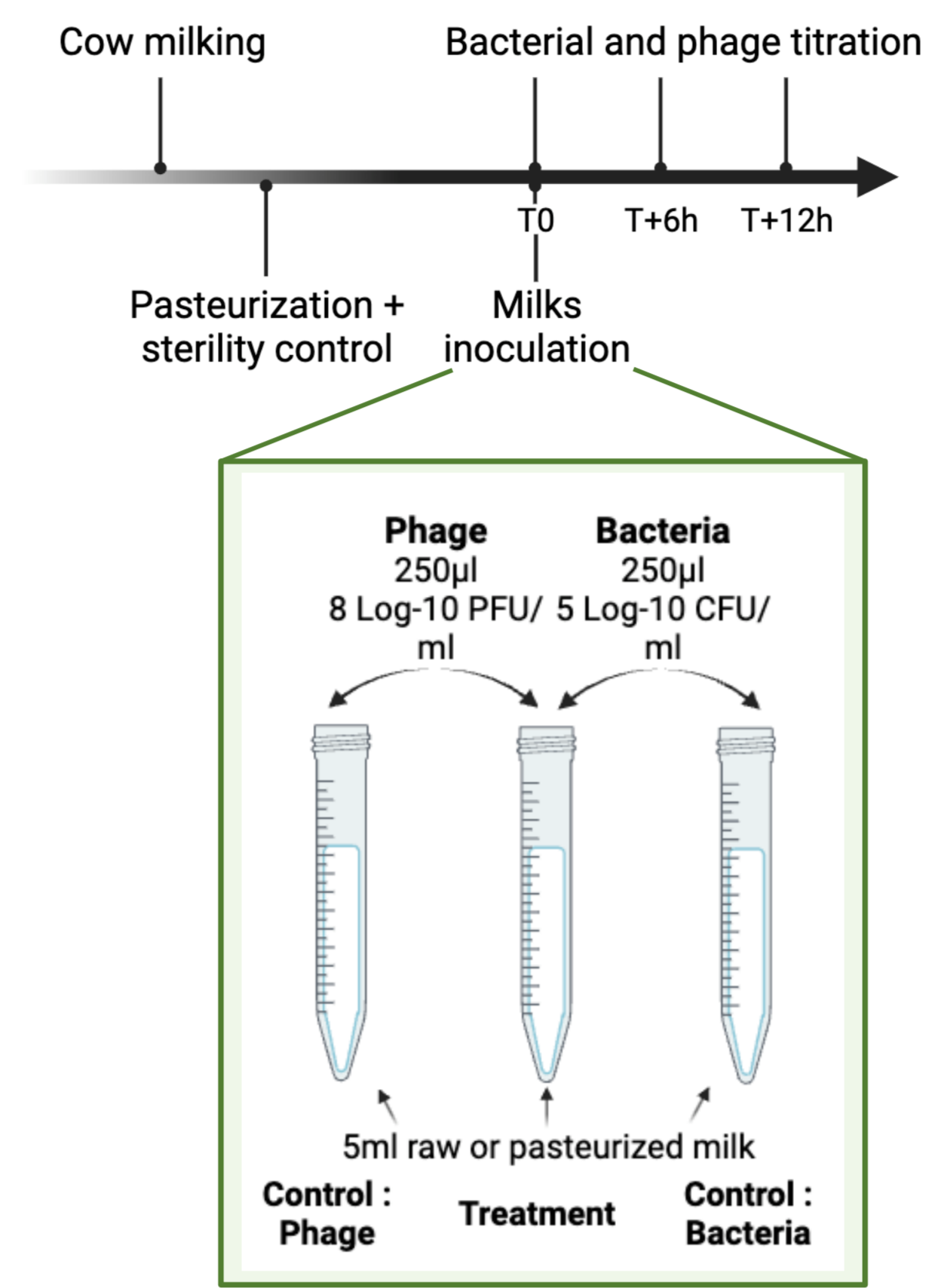
Figure 7. Optimal MOI

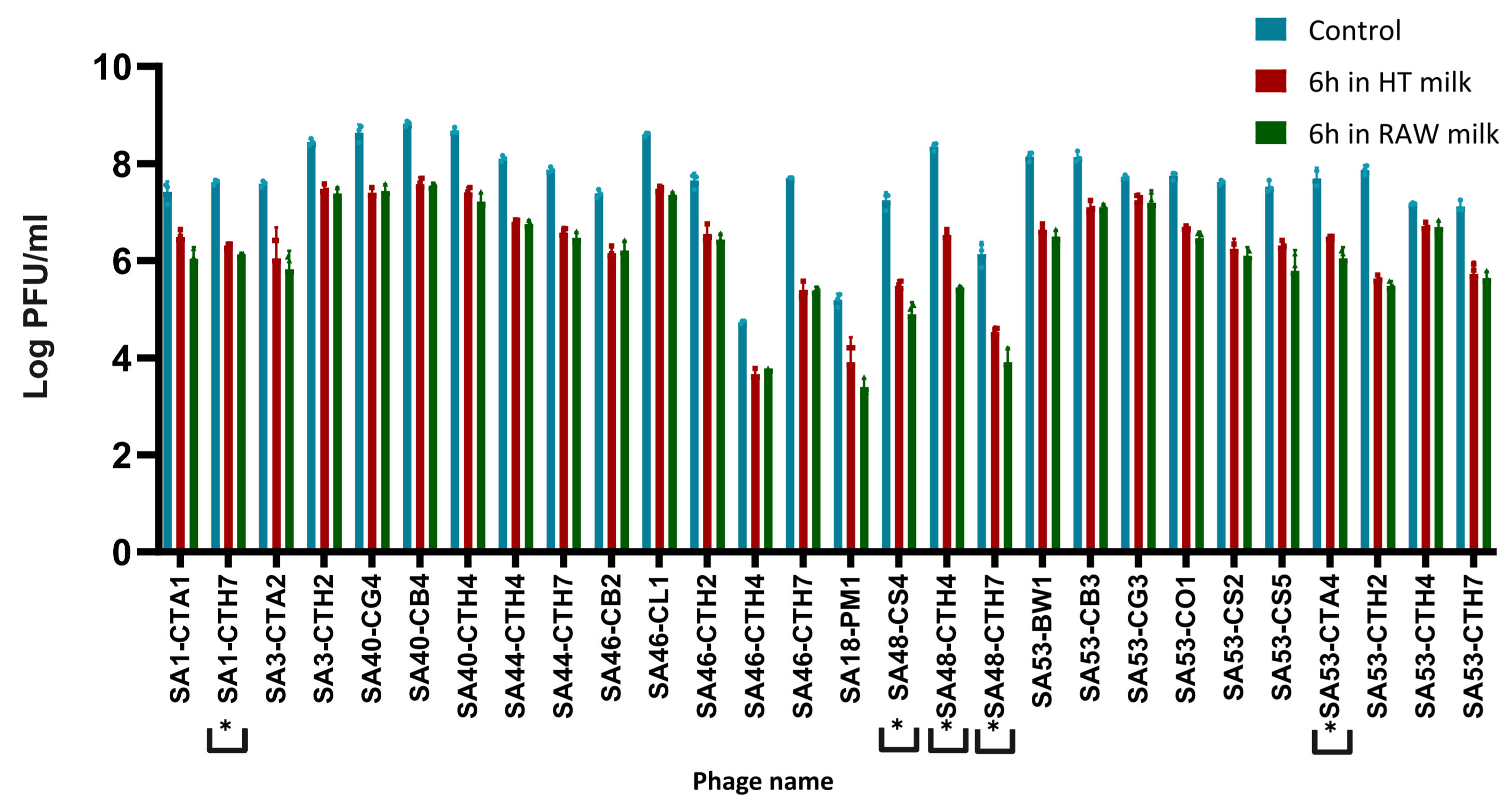
Conclusion

The use of bacteriophages for treating *Staphylococcus aureus* mastitis appears promising but our findings revealed several challenges that still need to be addressed. We have demonstrated the detrimental effects of milk on phages, particularly when incubated in raw milk. Specifically, raw milk showed a deleterious effect on phage stability. Furthermore, we demonstrated an increase in phage replication over time, albeit less pronounced in raw milk than in pasteurized milk. Inoculating milk with the phages optimal MOIs resulted in a constant decrease in bacterial count only in UHT. These observations may be due to proteins associated with milk fat globule membranes. These heat-sensitive components alter phage adsorption and reduce their efficiency. Other proteins like lactoperoxidase could also adversely affect phages and resist to the pasteurization process. The competition between bacteria and phages might promote bacterial mutations that confer resistance to the phages and could even engender faster-growing strains. As the effects described above hinder the ability of phages to kill mastitis associated *S. aureus*, formulation studies are needed to circumvent this issue.

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A: Pasteurized vs raw after 6h
 B: Pasteurized vs raw after 12h
 C: Pasteurized vs raw
 D: Effect of incubation time in raw milk
 E: Effect of incubation time in pasteurized milk

