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# The concerted action of lactoferrin and bacteriophages in the clearance of bacteria in sublethally infected mice*

## Laktoferryna wzmaga przeciwbakteryjne działanie suboptymalnych dawek bakteriofagów u infekowanych myszy

**Authors' Contribution:**
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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## Summary

**Background:** Both lactoferrin (LF) and bacteriophages are potent antibacterial agents. LF is contained in the secretory fluids of mammals and bacteriophages are specific bacterial viruses.

**Objectives:** The aim of this investigation was to determine whether combined treatment of infected mice may allow lowering the therapeutic dose of specific bacteriophages for *Escherichia coli* and *Staphylococcus aureus*.

**Materials/Methods:** CBA mice were infected intravenously (i.v.) with sublethal doses of *E. coli* or *S. aureus* and the specific T4 or A5 bacteriophages, respectively, were administered intraperitoneally (i.p.) or per os one hour following infection. The numbers of colony-forming units (CFUs) were determined in the livers after 24 hours.

**Results/Conclusions:** Comparative administration of bacteriophages *i.p.* or *per os* showed that both routes of administration were equally efficacious in the protective action of bacteriophages. The bacteriophages were still very potent in reducing CFU numbers in the liver at a dose of $10^5$/mouse. Application of bovine lactoferrin (LF), 10 mg i.v., 24 h before infection, was also very effective in reducing CFU numbers. Using suboptimal ($10^3$–$10^4$) doses of bacteriophages and administration of LF, a more potent protective effect in reducing the CFU numbers in the infected mice was demonstrated. The combined effect of LF and bacteriophages in reducing CFU numbers was significantly higher than the effects of either agent alone. The study demonstrated that the combined application of LF and bacteriophages can significantly lower (1000 times) the effective dose of bacteriophages in reducing CFU numbers in infected mice.

**Key words:** **lactoferrin • *Escherichia coli* • *Staphylococcus aureus* • bacteriophages • mice • CFU**

## Streszczenie

Zarówno laktoferryna (LF), jak i bakteriofagi należą do silnych czynników bakteriobójczych. Celem pracy było wykazanie, czy uprzednie podanie LF myszom zainfekowanym bakteriami pałeczki jelitowej lub gronkowcem złocistym pozwoli na obniżenie terapeutycznej dawki swoistych bakteriofagów dla tych bakterii. Myszy CBA były infekowane dożylnie subletalną dawką *Escherichia coli* lub *Staphylococcus aureus*. Po godzinie od infekcji myszom podano dootrzewnowo lub dożołądkowo swoiste bakteriofagi, odpowiednio T4 lub A5. Po upływie 24 godzin oznaczano liczbę bakterii w wątrobach zakażonych myszy jako liczbę kolonii na płytkach agarowych (CFU). Podanie bakteriofagów dootrzewnowo lub dożołądkowo było porównywalnie efektywne

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w usuwaniu bakterii z wątroby. Dawka bakteriofagów $10^5$/mysz redukowała liczbę CFU w wątrobie jeszcze w sposób bardzo znaczący. Zastosowanie wołowej LF (10 mg *i.v.*, na 24 godziny przed infekcją) było również bardzo efektywne w obniżeniu liczby CFU w wątrobie. Jednakże, stosując suboptymalne dawki bakteriofagów ($10^3$–$10^4$) i podanie LF, byliśmy w stanie wykazać znacznie silniejszy efekt bakteriobójczy niż wynikałoby to z sumarycznego działania tych czynników zastosowanych oddzielnie. W przypadku jednoczesnego zastosowania LF i bakteriofagów efektywną dawkę bakteriofagów można było obniżyć nawet 1000-krotnie.

**Słowa kluczowe:** **laktoferryna • *Escherichia coli* • *Staphylococcus aureus* • myszy • CFU**

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## Introduction

Phage therapy has attracted considerable interest associated with the phenomenon of increased resistance of pathogenic bacterial strains to antibiotics (reviewed in [3]). Treatment of patients with bacteriophages, both topically and orally, has proved very effective in combating suppurative infections [13]. Apart from a direct antibacterial action, bacteriophages can exhibit various beneficial effects on the function of the immune system. We recently demonstrated that effective phage therapy was associated with a normalization of cytokine production by blood cells isolated from patients [15]. Successful phage therapy also led to an acceleration of neutrophil turnover [16], although the ability of these cells to phagocytize bacteria was decreased. More recently we showed that bacteriophages can decrease the production of free radicals by human blood phagocytic cells [9]. Despite their specific and effective action, the oral application of bacteriophages has some limitations, since probably only a small fraction of the bacteriophages passes through the intestinal/blood barrier.

Lactoferrin (LF) is a protein found in the secretory fluids of mammals and the secondary granules of neutrophils (reviewed in [7]). The protein exhibits both direct [4,7,8] and indirect [6,17,18] antibacterial and anti-inflammatory actions in experimental endotoxemia. We demonstrated that the indirect anti-bacterial actions of LF in a mouse model was associated with triggering neutrophil release from the bone marrow [19]. LF was also shown to accelerate myelopoiesis in immunocompromised mice [1,2]. In human volunteers, oral administration of LF also enhanced the turnover of neutrophils [21].

Very recently we demonstrated in an individual with an ear infection that phage therapy was only partially effective, but an additional application of LF led to complete recovery [14]. Interestingly, LF treatment was accompanied with a long-term increase in both mature and immature neutrophil levels in the circulation and an elevation of endogenous LF concentration.

Taking into account the recently demonstrated antibacterial actions of LF, we hypothesized that combined treatment of experimental infections with bacteriophages and LF may result in a better therapeutic effect than treatment with bacteriophages or LF alone. In addition, we anticipated that the application of the combined therapy would allow a significant reduction in the number of administered bacteriophages.

## Materials and Methods

### Animals, strains and reagents

CBA mice of both sexes, 10–12 weeks old, were obtained from the Animal Facility in Ilkowice/Kraków, Poland. The mice had free access to water and standard rodent laboratory chow. All protocols were approved by the local ethics committee.

The bacterial strains (*Escherichia coli* B and *Staphylococcus aureus* L) were obtained from the collection of microorganism of the Institute of Immunology and Experimental Therapy, Wrocław, Poland. The virulent *E. coli* T4/coli B and *S. aureus* A5/L bacteriophages were from the same collection. Bovine lactoferrin (LF), endotoxin <4.4 U.E./mg, <25% iron saturated, was from Morinaga, Japan. The preparation and purification of the specific bacteriophages were described by us elsewhere [22].

### Treatment of mice with *Escherichia coli* and *Staphylococcus aureus*, bacteriophages, and lactoferrin

Mice were injected with the bacteria intravenously (i.v.) into the lateral tail vain in 0.2 ml of saline ($10^8$/mouse). Bacterial cell numbers were determined colorimetrically at a wavelength of 600 nm according to previously prepared standards. The virulent *E. coli* T4/coli B and *S. aureus* A5/L bacteriophages were administered intraperitoneally (i.p.) or orally using a stomach tube one hour after bacteria injection. Sterile phage lysates, which cause comple-

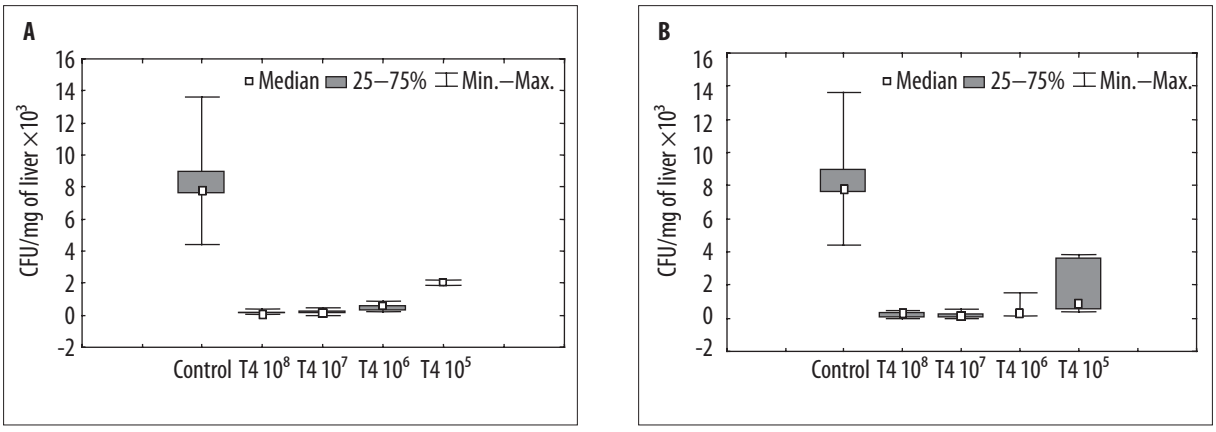


Figure 1. Efficacy of intraperitoneal and oral administration of T4 phages in the clearance of *E. coli* in the livers of infected mice. Mice were injected i.v. with $10^8$ *E. coli* (Control) and one hour later with $10^8$–$10^5$ T4 phages by the intraperitoneal (**A**) or oral (**B**) route. 24 h later the CFUs in the livers were enumerated. Significance: (**A**) Control vs. T4 $10^8$ $p$=0.0001, Control vs. T4 $10^7$ $p$=0.0001, Control vs. T4 $10^6$ $p$=0.0001, Control vs. T4 $10^5$ $p$=0.0001, T4 $10^6$ vs. T4 $10^5$ $p$=0.0002, T4 $10^7$ vs. T4 $10^5$ $p$=0.0001, T4 $10^8$ vs. T4 $10^5$ $p$=0.0001 (ANOVA); (**B**) Control vs. T4 $10^8$ $p$=0.0001, Control vs. T4 $10^7$ $p$=0.0001, Control vs. T4 $10^6$ $p$=0.0001, Control vs. T4 $10^5$ $p$=0.0001, T4 $10^7$ vs. T4 $10^5$ $p$=0.0216, T4 $10^8$ vs. T4 $10^5$ p=0.0411 (ANOVA)

te lysis of bacterial strains, were prepared according to Ślopek et al. [11]. Lactoferrin was injected i.v. into the retroorbital plexus at a dose of 10 mg/mouse 24 h before infection with bacteria.

### Determination of *E. coli* and *S. aureus* in the liver

Twenty-four hours after the injection of bacteria, the mice were sacrificed and the livers were isolated and homogenized using a plastic syringe piston and plastic screen, in sterile PBS (1 g of wet tissue per 25 ml of PBS). Five- and fifty-fold dilutions of cell suspension were applied onto McConkey and Chapmann agar plates and incubated overnight and the colony-forming units (CFU) were enumerated. The number of colonies was expressed as the number of CFUs per milligram of the organ.

### Statistical analysis

For statistical evaluation of the data, analysis of variance (ANOVA) or ANOVA of Kruskal-Wallis was applied. The Brown-Forsyth's test was used to determine the homogeneity of variance. Each experimental group consisted of seven mice. The results are presented as the median, 25–75% values, and min.-max. values and were regarded to be significant when p<0.05.

## Results

### Comparison of the efficacy of intraperitoneal and oral T4 bacteriophage treatment in the experimental infection with *Escherichia coli*

Oral phage therapy has proved to be effective in patients bearing various types of external and internal infections [3,11,13,15]. The aim of the present experiment was to determine how effective oral treatment of mice infected with *E. coli* might be in comparison with intraperitoneal administration of T4 bacteriophage. The bacteriophages were administered in a dose range of $10^8$–$10^5$ one hour after i.v. infection of the mice with bacteria. Twenty-four hours after infection, the number of CFUs in the liver was counted. Figure 1AB shows that both ways of bacteriophage treatment appeared to be equally effective in reducing CFU numbers in the liver. Although the $10^7$ dose resulted in a statistically significant effect, the $10^5$ dose still gave marked inhibition.

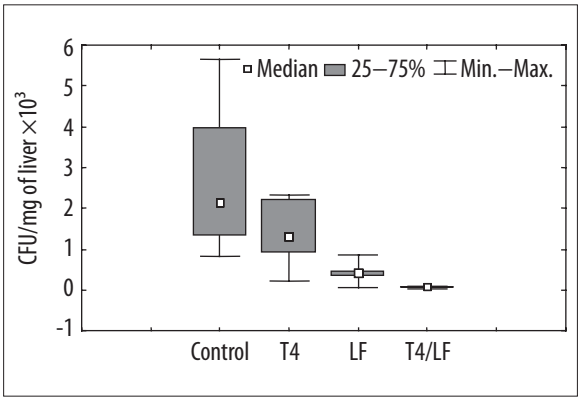


Figure 2. Cooperative action of lactoferrin and T4 phages in the clearance of *E. coli* in the livers of infected mice. Mice were given LF i.p. (10 mg/mouse). 24 h later the animals were infected i.v. with $6.7\times10^8$ *E. coli* and after one hour with $10^3$ phages *per os*. Control mice were injected only with *E. coli*. The number of CFU in the livers was determined 2 h later. Significance: Control vs. LF $p$=0.0357, Control vs. T4/LF $p$=0.0000, T4 vs. T4/LF $p$=0.0036 (ANOVA of Kruskal-Wallis)

### Cooperation of lactoferrin and T4 bacteriophages in the clearance of *E. coli* in the livers of infected mice

Mice were given LF i.v. 24 h before infection. T4 bacteriophages were administered at a suboptimal dose of $10^3$ i.p. one hour after infection. The results of this experiment are shown in Figure 2. The results show that although treatment with bacteriophages T4 or LF alone led to reductions in the CFU numbers in the liver, the combined effect of bacteriophages and LF produced a significant additive effect.

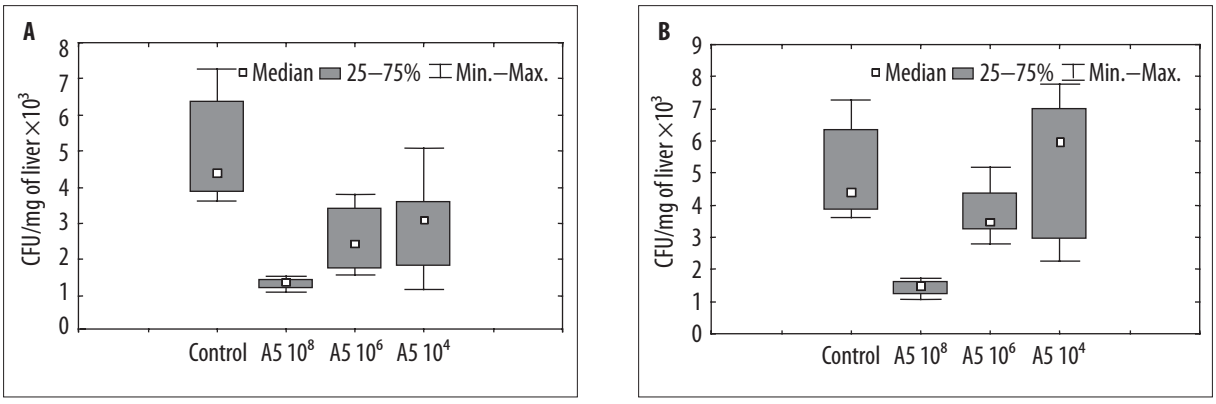


Figure 3. Efficacy of intraperitoneal and oral administration of A5 phages in the clearance of *S. aureus* in infected mice. Mice were injected i.v. with $2\times10^7$ *S. aureus* (Control) and one hour later with $10^8$–$10^4$ of A5 phage intraperitoneally (**A**) or *per os* (**B**). 24 h later the CFU numbers in the livers were determined. Significance: (**A**) Control vs. A5 $10^8$ $p$=0.0002, Control vs. A5 $10^6$ $p$=0.0082, Control vs. A5 $10^4$ $p$=0.0312 (ANOVA); (**B**) Control vs. A5 $10^8$ $p$=0.0065 (ANOVA of Kruskal-Wallis)

### Comparison of the efficacy of intraperitoneal and oral A5 bacteriophages treatment in combating *Staphylococcus aureus* infection

The efficacy of oral treatment of mice infected with *S. aureus* in comparison with the i.p. route of bacteriophage administration is presented in Figure 3AB. The bacteriophages were used at a dose range of $10^8$–$10^4$/mouse. The results show a dose-dependent inhibition of CFU numbers in the livers, similar in both routes of bacteriophage administration. However, a statistically significant effect was observed only at the $10^8$ bacteriophage dose. Inhibition at the $10^6$ bacteriophage dose was less evident and $10^4$ was not effective.

### Cooperation of lactoferrin and A5 bacteriophages in the clearance of *S. aureus* in the livers of infected mice

The combined protective effect of lactoferrin and bacteriophage treatment in *S. aureus* infection of mice is shown in Figure 4. Bacteriophages given at doses of $10^5$ and $10^3$ or the treatment with LF appeared to be markedly inhibitory in reducing CFU numbers in the liver. However, the combined treatment of mice with LF and bacteriophages resulted in a further, significant decrease in CFU numbers.

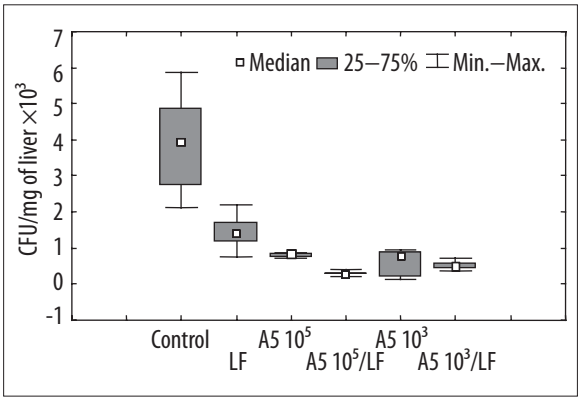


Figure 4. Cooperative action of lactoferrin and A5 phages in the clearance of *S. aureus* in the livers of infected mice. Mice were given LF i.p. (10 mg/mouse) and infected i.v. 24 h later with $10^8$ *S. aureus*. A5 phages were administered *per os* one hour following infection. Control mice were injected only with *S. aureus*. 24 h later the CFU numbers in the livers were determined. Significance: Control vs. A5 $10^5$/LF $p$=0.0000, Control vs. A5 $10^3$/LF $p$=0.0001, LF vs. A5 $10^5$/LF $p$=0.0002, LF vs. A5 $10^3$/LF $p$=0.0186 (ANOVA of Kruskal-Wallis)

## Discussion

The presented results demonstrated that oral treatment of mice infected with Gram-positive or Gram-negative bacteria with specific bacteriophages was effective in reducing CFU numbers in the livers to a similar degree as intraperitoneal administration of bacteriophages. In addition, pretreatment of these mice with lactoferrin resulted in a more effective destruction of bacteria and allowed a significant reduction in the number of applied phages.

The efficacy of the oral treatment of mice with phages was not surprising since the penetration of phages into the circulation by the gastric route is rapid [5]. However, remarkably low ($10^4$–$10^3$/mouse) orally administered doses of phages were able to significantly reduce the number of CFUs in the livers of infected mice (data not shown). We also demonstrated that the staphylococcal A5 phage was able to provide protection against *S. aureus* in a fashion similar to that of T4 phage specific to *E. coli*, which contradicts another study showing no efficacy of a staphylococcal phage in experimental infection [12]. The efficacy of the oral treatment of infected mice with specific bacteriophages may explain the successful oral phage therapy in patients.

The advantages of phage therapy compared with antibiotic therapy are obvious, since bacteriophages selectively destroy pathogenic bacterial strains without any harmful bystander effects. Our recent studies showed that the administration of purified phage preparations could create favorable conditions for combating bacterial infections while at the same time protecting the host's cells from damage. For example, IL-6 production was increased without elevation of TNF-alpha production [20]. Also, phages were found to reduce free-radi-

cal production in phagocytic cells [9]. In addition, bacteriophages, apart from their direct destructive effect on bacterial cells, may facilitate the uptake of bacteria by phagocytes, probably by means of specific opsonization [9].

The ability of LF to enhance the clearance of bacteria in organs is primarily associated with the release of neutrophils, the major phagocytes, from the bone marrow into the circulation [19]. The regulation of cytokine release during bacterial infection by LF [19] or serum iron withholding [17] may also be of importance in the more efficient disposal of pathogens. So far only one observation regarding a complementary action of bacteriophages and orally administered LF in bacterial inflammation has been reported [14]. The treatment of the patient with LF led to characteristic changes in some circulatory blood parameters, such as a long-term increase in the level of endogenous LF and an acceleration of neutrophil turnover as reflected by an increased level of neutrophil precursors.

The experimental protocol applied in this study was intentionally designed to facilitate the clearance of bacteria by administration of LF prior to infection and the application of phages. In such a protocol the injected bacteria are most likely confronted with substantially higher neutrophil numbers [19] able to eliminate them more efficiently. It is, however, possible that a concomitant or delayed administration of LF would be also beneficial in accelerating the removal of bacteria by bacteriophages, and such protocols will be applied in our future studies.

In summary, this preliminary investigation demonstrated that experimental bacterial infections in mice can be more successfully eliminated using a combined treatment of animals with LF and specific bacteriophages. Such an approach enables a significant reduction in the number of administered phages, probably due to the rapid release of neutrophils from the bone marrow reservoir by lactoferrin. Further investigations are, however, required to examine the efficacy of other experimental protocols involving the combined application of LF and bacteriophages.

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