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EXPERIMENTAL ARTICLES

Investigation of Lysozyme–Antilysozyme Interactions in a Model *Tetrahymena–Escherichia* Community

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Abstract—Lysozyme and antilysozyme activities present in a wide range of microorganisms determine the so-called lysozyme–antilysozyme system of hydrobionts, which greatly contribute to the formation of aquatic biocenoses. However, the mechanism of the functioning of this system in natural freshwater communities remains obscure. The experimental investigation of lysozyme–antilysozyme interactions in a model *Tetrahymena–Escherichia* community showed that the antilysozyme activity of *Escherichia coli* leads to incomplete phagocytosis, thus enhancing bacterial survival in a mixed culture with infusoria. The selection and reproduction of bacterial cells resistant to grazing by infusoria determine the character of host–parasite interactions and allow bacteria to survive. It was demonstrated that the antilysozyme activity of microorganisms, which is responsible for bacterial persistency in natural biocenoses, is involved in the maintenance of protozoa–bacteria communities in bodies of water.

Key words: protozoans, bacteria, lysozyme, antilysozyme, aquatic biocenoses.

Lysozyme (muramidase) is widespread in plants, animals, and microorganisms [1] Some microorganisms are able to inactivate lysozyme; i.e., they possess antilysozyme activity [2]. The functional lysozyme–antilysozyme system of hydrobionts plays an important role in the formation of aquatic biocenoses [3]. However, the mechanism of the functioning of this system in natural communities of freshwater bodies has not yet been investigated.

The present work was undertaken to study lysozyme–antilysozyme interactions in a model protozoan–bacterium community.

MATERIALS AND METHODS

Two isogenic *Escherichia coli* K-12 strains used in this work were a generous gift from V.M. Bondarenko, Gamaleya Research Institute of Epidemiology and Microbiology in Moscow. Unlike *E. coli* K-12 J53 (ALA^-), the strain *E. coli* K-12 J53 tr 22-110 (ALA^+) carried a plasmid that imparted antilysozyme activity to this strain. The axenic culture of the infusorian *Tetrahymena pyriformis* (strain GL) was maintained by subculturing it in 0.3% heart–brain broth (Difco) once a week in a ratio of 0.1 ml culture to 10.0 ml medium. The infusorian was cultivated at 22–25°C. Three-day-old infusorial cultures (5 ml) containing 10^4 cells/ml were infected with the isogenic strains *E. coli* K-12 J53 tr 22-110 (experiment) or *E. coli* K-12 J53 (control) in a dose of 10^7 CFU/ml. The experimental and control cocultures were incubated at 22–25°C and examined on the 1st, 5th, 10th, 15th, 20th, 30th, and 40th days of incubation. To this end, infected infusorial cells were washed off of nonpenetrated bacterial cells by repeated centrifugation in cold (4°C) 0.2 M phosphate buffer, and the infusorial cells were counted using a Goryaev–Thoma chamber. The lysozyme activity of the protozoan was assayed nephelometrically as described elsewhere [4]. Bacteriological tests were carried out by plating the appropriate culture dilutions onto nutrient and Endo agar media. The antilysozyme activity of *E. coli* was assayed as described earlier [2]. The experiment lasted 40 days. The data obtained were statistically processed as described by Kaminskii [6].

Electron microscopic studies were carried out in the early terms of infection (1 to 24 h after mixing the *E. coli* and *T. pyriformis* cultures). Specimens were prepared by standard techniques [5]. Specifically, the infected infusoria were separated from the nonpenetrated bacteria by filtration through 5-μm-pore-size Millipore filters, washed with a cold (4°C) 0.2 M cacodylate buffer (pH 7.2), fixed using 4% glutaraldehyde and 1% osmium tetroxide, dehydrated in a series of alcohol solutions of increasing concentration, and embedded in epoxy resin. The specimens thus prepared were examined in a JEM-100B electron microscope (Japan) at a magnification of 3000 to 54000× in no fewer than 100 microscopic fields.

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The electron microscopic studies were carried out in the laboratory of the anatomy of microorganisms at the Gamaleya Institute of Epidemiology and Microbiology.

RESULTS AND DISCUSSION

Lysozyme–antilysozyme interactions were studied using the cocultures of infusoria with two isogenic strains *E. coli* J53, either possessing or not antilysozyme activity (ALA^+ and ALA^- phenotypes, respectively). In the control coculture, the concentration of *E. coli* (ALA^-) cells decreased from 10^7 to 8×10^2 CFU/ml in the first day, to 10^2 CFU/ml by the 10th day, and to 10^1 CFU/ml by the 30th day of incubation. After 35 days of the experiment, *E. coli* cells were not detected at all, probably because of their complete grazing by infusoria. The number of infusorial cells in the control coculture remained at a level of 1×10^4–1×10^3 cells/ml.

In the experimental coculture, the concentration of *E. coli* (ALA^+) cells decreased from 10^7 to 10^4 CFU/ml in the first day and then gradually increased to make up 5×10^5 CFU/ml by the 15th day of incubation. By the 20th day, the concentration of *E. coli* (ALA^+) stabilized at a level of 10^4 CFU/ml and did not change for the rest of the experiment. The number of infusorial cells in the experimental coculture remained at a level of 10^4 cells/ml within the first 10 days, decreasing to 10^3 cells/ml by the 40th day of incubation (Fig. 1).

After 20 days of cocultivation on Endo medium, the *E. coli* (ALA^+) strain produced, along with large ($d \approx 3$ mm) smooth colonies with a typical metallic lustre, small ($d \approx 1$ mm) smooth colonies. Cells taken from such small colonies slowly fermented lactose and had a slightly higher antilysozyme activity (5–6 μg/ml) than the *E. coli* (ALA^+) cells taken from the large colonies (2–4 μg/ml). It should be noted that the formation of small colonies was not observed in the control coculture of the *E. coli* (ALA^-) strain with infusoria.

The level of endogenous lysozyme in the infusoria cocultivated with *E. coli* (ALA^-) cells decreased from 1.19 ± 0.06 μg/ml at the beginning of the experiment to 0.8 ± 0.10 μg/ml at the end of the experiment. The same decrease in the level of endogenous lysozyme (from 1.2 ± 0.06 to 0.7 ± 0.10 μg/ml) was observed in a monoculture of infusoria.

In the infusoria cocultivated with the *E. coli* (ALA^+) cells, the level of endogenous lysozyme decreased from 1.19 ± 0.14 μg/ml to 0.2 ± 0.01 μg/ml ($p < 0.05$) by the 15th day and to almost zero by the 20th of cocultivation. Taking into account the presence of living infusoria in the coculture throughout the incubation period, these data can be interpreted as the blocking of infusorial lysozyme by bacterial antilysozyme.

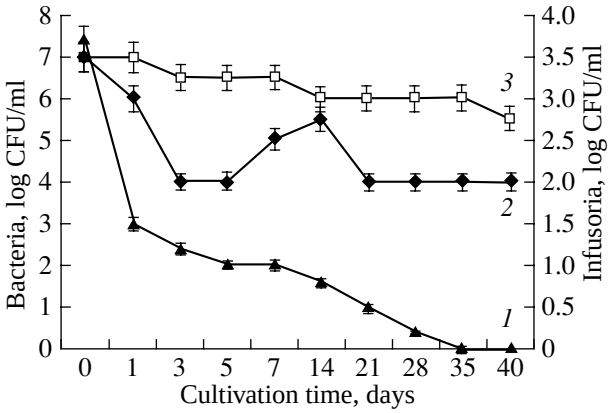

Fig. 1. The population dynamics of (*1*) *E. coli* (ALA^-) and (*2*) *E. coli* (ALA^+) in cocultures with (*3*) infusoria.

The antilysozyme activity of *E. coli* (ALA^+) cells isolated from infusoria 1 day after the beginning of the experiment was 2.8 ± 0.20 μg/ml as compared to a value of 3.0 ± 0.10 μg/ml observed prior to cocultivation. In the next 9 days, the antilysozyme activity of *E. coli* cells increased to 3.7 ± 0.2 μg/ml ($p < 0.05$). Then the antilysozyme activity gradually declined to make up 3.2 ± 0.25 μg/ml by the 40th day of cocultivation.

The population analysis of the bacterium–protozoan cocultures showed that the concentration of *E. coli* (ALA^+) cells remained at a sufficiently high level throughout the period of the experiment (40 days), whereas *E. coli* (ALA^-) cells in the control coculture could not be detected as early as after 20 days of cocultivation. The mechanism of this phenomenon is as yet unclear. In view of this, we undertook electron microscopic studies of the early stages of protozoan infection with bacteria, which most adequately reflect the process of phagocytosis [7].

Ultrastructural changes in the *E. coli* (ALA^+) cells occurring in the phagosomes (digestive vacuoles) of infusoria were studied in dynamics (after 1, 3, 6, and 24 h of cocultivation).

The electron microscopic investigations of specimens prepared from a 1-h- old coculture of *E. coli* cells with infusoria (Fig. 2) revealed a great number of phagosomes occurring primarily in the region of the infusorial peristome, some of which contained single bacterial cells and the others contained 50–60 bacterial cells. The absorbed bacterial cells did not differ ultrastructurally from intact cells. However, the accumulation of lysosomal granules and mitochondria around the vacuole was indicative of the beginning of the digestion of absorbed bacterial cells (Fig. 3).

After 3 and 6 h of cocultivation, the phagolysomes containing *E. coli* cells at different stages of digestion

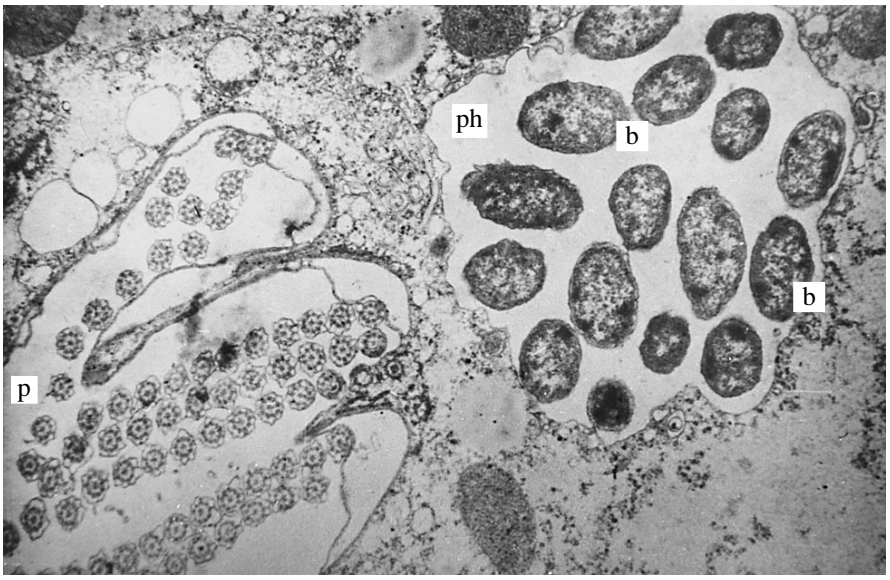

Fig. 2. A phagosome containing intact bacterial cells after 1 h of the coincubation of bacteria with infusoria (ph, phagosome; p, peristome; and b, bacteria). Magnification, 16000×.

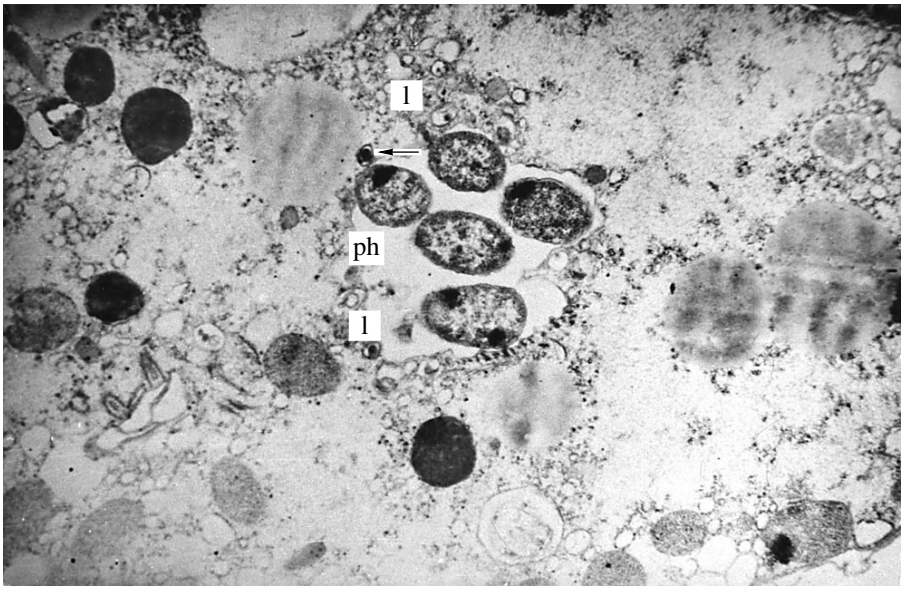

Fig. 3. Accumulation of lysosomes around a phagosome after 1 h of the coincubation of bacteria with infusoria (l, lysosomes). Magnification, 13500×.

could be observed in one infusorial cell. Some phagosomes contained dense bacterial cells of a modified form, whereas bacterial cells in other phagosomes were completely digested (Fig. 5). As a rule, the phagosomes also contained 2–4 intact bacterial cells bounded by multilayer myelinlike structures. The phagosomes of the infusoria coincubated for 6 h with the *E. coli* (ALA^-) strain contained altered bacterial cells or their fragments (Fig. 4).

Most phagosomes of the infusorial cells incubated with the *E. coli* (ALA^+) cells for 1 day contained fragments of completely digested bacterial cells, whereas other phagosomes contained bacterial cells at different stages of digestion, as well as single cells resistant to digestion and structurally looking like intact *E. coli* (ALA^+) cells (Fig. 6). In the control coculture, the process of phagocytosis was fully completed within 1 day of coincubation, as is evident from the absence of phagosomes and bacterial cells in the infusoria (Fig. 7).

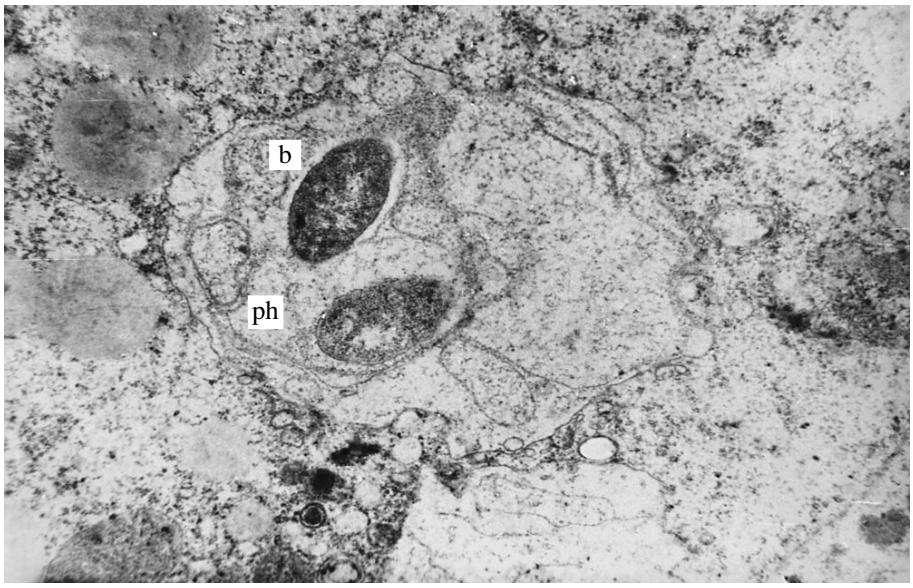

Fig. 4. A phagosome with intact bacterial cells after 6 h of the coincubation of bacteria with infusoria. Magnification, 16000×.

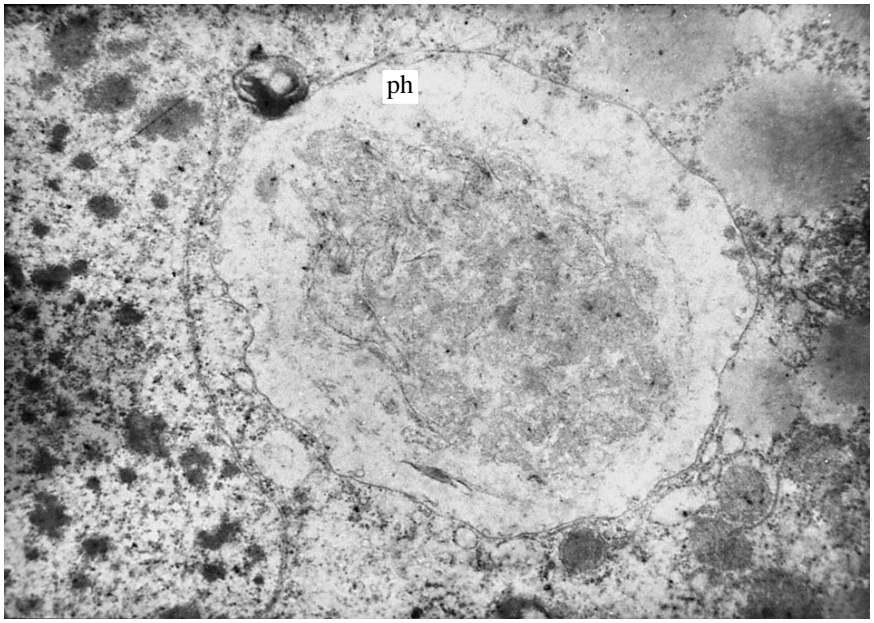

Fig. 5. A region of the infusorial endoplasm containing the remains of lysed *E. coli* (ALA^-) cells after 6 h of the coincubation of bacteria with infusoria. Magnification, 48000×.

The data obtained show that microorganisms possessing antilysozyme activity can survive in protozoa. The incomplete phagocytosis of *E. coli* (ALA^+) cells in the infusoria suggests the existence of a selective mechanism for the maintenance of the antilysozyme activity of bacteria during their interaction with protozoa, similar to the mechanism maintaining bacterial virulence in soil and aquatic biotopes [7].

The functional lysozyme–antilysozyme system, which is shown to be essential in the survival of *E. coli*, can probably be a universal system operating in human and animal organisms [8, 9], as well as in natural bodies of water [3].

It is believed that protozoa play a key role in the population ecology of bacteria in the environment [10]. Particularly, they may be involved in the selection of

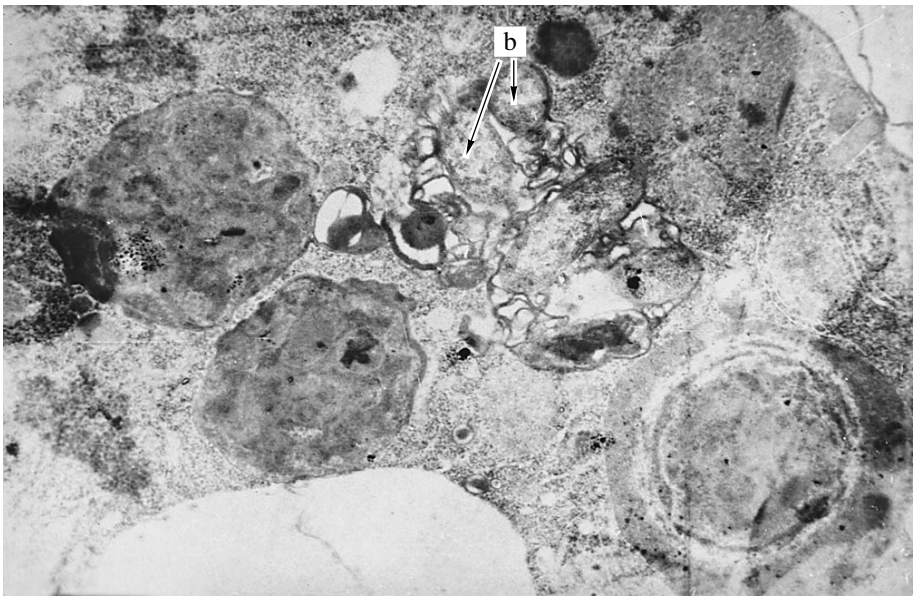

Fig. 6. The infusorial endoplasm containing phagosomes with more or less digested and intact (↓) bacterial cells after 24 h of the coincubation of bacteria with infusoria. Magnification, 13500×.

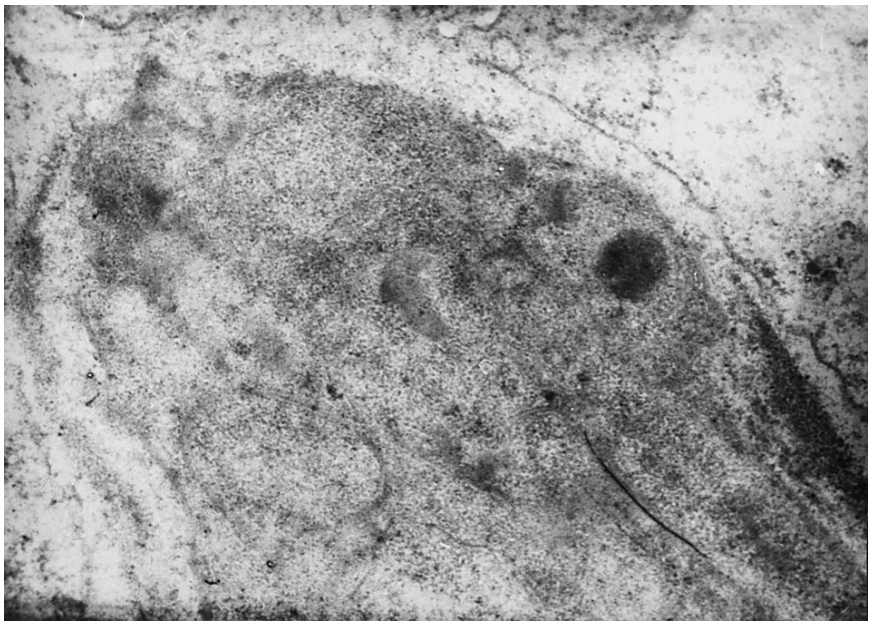

Fig. 7. The infusorial endoplasm free of bacteria and phagosomes after 24 h of the coincubation of *E. coli* (ALA^-) cells with infusoria. Magnification, 48000×.

more persistent clones of *E. coli* (ALA^+), thereby unfavorably influencing the sanitary ecology of bodies of water.

In conclusion, the antilysozyme activity of microorganisms protects them from protozoa and thus contributes to their survival in natural biocenoses. The lysozyme– antilysozyme interactions of hydrobionts may be considered one of the possible regulatory mechanisms of protozoa–bacteria communities in bodies of water, which enhance their stability.

REFERENCES

1. Bukharin, O.V., Vasil'ev, N.V., and Usvyatsov, B.Ya., *Lizotsim mikroorganizmov* (Microbial Lysozyme), Tomsk, 1985, pp. 6–16 and 174–183.

2. Bukharin, O.V., Usvyatsov, B.Ya., Malyshkin, A.P., and Nemtseva, N.V., Determination of the Antilysozyme Activity of Microorganisms, *Zh. Mikrobiol. Epidemiol. Immunobiol.*, 1984, no. 2, pp. 27–28.
3. Solovykh, G.N. and Nemtseva, N.V., The Lysozyme–Antilysozyme System of Hydrobionts and Its Role in the Formation of Aquatic Biocenoses, *Zh. Mikrobiol. Epidemiol. Immunobiol.*, 1994, no. 5 (Appendix), pp. 95–98.
4. Bukharin, O.V. and Vasil'ev, N.V., *Lizotsim i ego rol' v biologii i meditsine* (Lysozyme and Its Role in Biology and Medicine), Tomsk: Tomskii Univ., 1974, pp. 37–42.
5. *Manual of Methods for General Bacteriology*, Gerhardt, P. *et al.*, Eds., Washington: Am. Soc. Microbiol., 1981. Translated under the title *Metody obshchei bakteriologii*, Moscow: Mir, 1983, vol. 1, pp. 90–137.
6. Kaminskii, L.S., *Statisticheskaya obrabotka laboratornykh i klinicheskikh dannykh* (Statistical Processing of Laboratory and Clinical Data), Moscow: Meditsyna, 1964, pp. 61–217.
7. Litvin, V.Yu. and Pushkareva, V.I., About a Putative Mechanism for the Maintenance of Bacterial Virulence in Soil and Aquatic Communities, *Patogennye bakterii v soobshchestvakh* (Pathogenic Bacteria in Communities), Moscow, 1994, pp. 24–34.
8. Pruss, V.F., The Prognosis of Convalescent Carriage during Dysentery, *Persistentsiya mikroorganizmov* (Microbial Persistence), Kuibyshev: KMI, 1987, pp. 79–85.
9. Zarifullina, L.A., Antilysozyme Activity of Meningococci, *Cand. Sci. (Med.) Dissertation*, Chelyabinsk, 1986, pp. 10–15.
10. Jeon, K.W., Symbiogenesis of Bacteria within Amoebae, *Enigmatic Microorganisms and Life in Extreme Environment*, Seckbach, J., Ed., 1999, pp. 587–598.
11. Bukharin, O.V. and Litvin, V.Yu., *Patogennye bakterii v prirodnykh ekosistemakh* (Pathogenic Bacteria in Natural Ecosystems), Ekaterinburg, Ural Otd. Ross. Akad. Nauk, 1997, pp. 112–133.