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EXPERIMENTAL ARTICLES

# Morphometric Analysis of Bacteria Associated with Soil Millipedes

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**Abstract**—Scanning electron microscopy revealed that the average cell size of bacteria associated with the digestive tract of soil millipedes was 0.65 μm in diameter, 1.36 μm in length, and 0.60 $\mu m^3$ in volume. An example of millipedes illustrated that the intestinal tract bacteria of soil invertebrates share the following features: (1) a high density level in this habitat; (2) existence mostly in the form of vegetative cells; (3) a cell size significantly smaller than that of bacteria functioning in soil; (4) a cell size closer to the lower limits of the size range characteristic for bacterial cultures grown in laboratory media. All this suggests that the bacterial community of the digestive tract differs from the typical soil community not only in composition but also in a higher level of physiological activity.

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*Key words*: bacteria, millipedes, soil, microscopy.

That invertebrates play an important role in decomposing dead vegetable material is generally acknowledged [1]. However, invertebrates are only directly involved in 1 to 10% of the carbon flow that passes mainly through populations of microorganisms [2]. Therefore, the role of invertebrates in the saprotrophic process is usually associated rather with medium-forming activity for microorganisms than with their direct involvement in the utilization of organic compounds [3]. It was established that the food substrate in the intestines of invertebrates is ground and mixed, alkalinized and moistened, special redox conditions are provided for enzymes [1, 4], and the excretory products of nitrogenous metabolism are supplemented [5].

The composition and role of microorganisms in the digestive tract of soil invertebrates have not yet been studied in detail. Most of the relevant evidence was obtained by an indirect method of microbiological inoculation, enumerating only a part of the microbial populations. The method of scanning electron microscopy offers unique possibilities for the investigation of microorganisms in situ. The combination of high resolving power and a wide field of view, together with the possibility to examine massive and nontransparent objects, allows valuable and reliable information concerning the activity of microorganisms in their natural habitats to be obtained [6].

The aim of these studies was to reveal the specific features of the bacterial community associated with soil millipedes using scanning electron microscopy.

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## MATERIALS AND METHODS

The objects of this study were bacterial cells which were developed in the intestines and excrement of millipedes. The millipedes were collected in the litter of forest and forest–steppe ecosystems; *Pachyiulus flavipes* C.L. Koch was from brown soil under the broad-leaved forest near Gurzuf, Ukraine; *Glomeris connexa* C.L. Koch, *Leptoiulus polonicus* Jawlowski, and *Megaphyllum projectum* (Verhoeff) were from brown soil under the broad-leaved forest near Mukachevo, Ukraine; *Megaphyllum rossicum* (Tim) was from a typical chernozem under the broad-leaved forest in Kursk oblast; and *Megaphyllum rossicum* (Tim) and *Rossiulus kessleri* (Lohm.) were from a typical chernozem under acacia plants in the Rostov oblast.

Under laboratory conditions, the millipedes were kept in natural substrates in groups of 30 to 50 per 100 g of substrate in the desiccators at room temperature. The millipedes were then killed by immersing them in boiling water for 1 s or by decapitation. They were dissected under sterile conditions, and their digestive tracts were excised. The tract was then cut into fragments, washed in sterile tap water, and then dried either in the air or by the critical point method. The specimens were then attached to preparative tables with the D-550 conducting glue, their inner surface on the outside. Using a JSM-2 scanning electron microscope, the epithelium of the middle intestinal division and the cuticle of the anterior and posterior intestinal divisions, as well as the inner surface of the peritrophic membrane, were examined. The average dry mass of a millipede digestive tract was from 1 to 10 mg.

The fresh excrement of the millipedes were air-dried, glued onto preparative tables, and their outer surface was examined. The dry mass of one excrement constituted 1 to 2 mg.

The size of the bacteria was measured in the samples, from the chernozem soils from Kursk, Voronezh, and Samara oblasts, and of the soddy podzolic soils from Moscow, Perm', and Bryansk oblasts. Each of the soils studied was represented by several samples of the upper humus-containing horizon (5–20 cm). The development of heterotrophic microorganisms was initiated by the introduction of various organic substrates (plant offal, potato starch, glucose, and certain other carbohydrates); the surface of soil plates was examined under laboratory conditions (25°C, field moisture capacity). A detailed description of the methods of electron microscopic study of soils and invertebrates was given earlier [6–8].

The bacteria were photographed, and when the cell length and diameter measurements were measured on the prints; the image scale was taken into account. Only cells, which exhibited distinct signs of colonial growth, were considered; single cells were excluded. The goal of this procedure was to ensure enumeration mostly of the microorganisms functioning under soil conditions, while excluding allochthonous microorganisms. The results were statistically processed using the Statistica software package.

## RESULTS

**Morphological diversity of bacterial cells**. The cell size of the bacteria associated with soil millipedes and of those that independently developed in soil were determined using a scanning electron microscope. The micrographs (Fig. 1) illustrate the diversity of the cell shape of bacteria revealed in the millipede intestines and excrement, as well as the population density of this habitat; coccoid, oval, rod-shaped, and filamentous cells occurred. The cocci differed both in their size and by the number of division planes (Fig. 1a); the rods differed in size, diameter to length ratio, and curvature (Figs. 1a, 1b). In aggregations, rods sometimes formed branching structures resembling pseudomycelium (Fig. 1c). Fine coenocytic hyphae and the hyphae fragmenting into individual rods were revealed (Fig. 1d). It should be noted that, in millipede excrement, the microbial cells sometimes appeared to be completely immersed in a mucous mass (Fig. 1b). This method, therefore, does not allow for the exact qualitative characteristics of the microbial population density to be obtained. However, as seen in the micrographs presented, the bacteria form multilayered aggregations in some loci along the digestive tract, while only individual microcolonies occurred in other parts. All this allows us to consider this habitat as eutrophic with a high population density.

While drying, the excrement sometimes revealed new crystalline formations (Fig. 1d), which, though indirectly, give evidence to a rate of the mineralization of deciduous plant material in the digestive tract of soil invertebrates. These formations are most likely derived from the excrement of nitrogen metabolism.

**Ranking of bacterial cells by size.** Bacterial cells (2911) associated with millipedes, including intestinal and excrement bacteria, were measured. The range of cell diameter was from 0.18 to 2.30 μm; cell length (excluding mycelial organisms) ranged from 0.20 to 4.40 μm. Ranking this set into a variation series with a 0.5 μmlength of the class interval (Fig. 2a) allowed a three-dimensional dome-shaped histogram with a single apex corresponding to the maximum rate of occurrence of the cells 0.5–1.0 μm in diameter and 1.0–1.5 μm in length to be obtained. The average cell size was 0.65 ± 0.01 μm in diameter, 1.36 ± 0.02 μm in length, and $0.60 \pm 0.02$ μm$^3$ in volume.

2623 bacterial cells whose active functioning was directly connected with soil were studied (Fig. 2b). The range of their diameter was 0.2–2.5 μm, and their length (excluding mycelial organisms) ranged from 0.3–7.3 μm. The cell-size distribution histogram was markedly dome-shaped with a maximum rate of occurrence for the cells 0.5–1.0 μm in diameter and 1.0–1.5 μm in length. The average bacterial cell measured $0.85 \pm 0.01 \times 1.54 \pm 0.02$ μm; its average volume was $0.94 \pm 0.02$ μm$^3$. A comparison between microorganisms from different habitats revealed that the distinctions occurred, mainly in the first horizontal row, represents the group of bacteria with diameters not more than 0.5 μm. In the subset of millipede-associated bacteria, it accounts for 48%, whereas in the group of soil bacteria, it accounts for less than 15%.

The 1917 bacterial cells from the intestines of invertebrates ranged from 0.2 to 2.0 μm in diameter and from 0.2 to 4.4 μm in length. Ranking this subset into a variation series with a length of the class interval also of 0.5 μm (Fig. 3a), resulted in a three-dimensional dome-shaped histogram, with an apex corresponding to the maximum rate of occurrence for cells 0.5–1.0 μm in diameter and 1.0–1.5 μm in length. The average cell size was 0.63 ± 0.02 μm in diameter, 1.40 ± 0.02 μm in length, and $0.64 \pm 0.01$ μm$^3$ in volume.

The distribution histogram by the cell size of 994 bacteria revealed that the excrement of millipedes was similar (Fig. 3b). The average cell size was 0.69 ± 0.01 in diameter, $1.30 \pm 0.02$ μm in length, and $0.55 \pm 0.03$ μm$^3$ in volume.

The size characteristics of the hyphae of actinomycete mycelium are presented separately (Fig. 4). The hyphal diameter of actinomycetes in soil varied from 0.30 to 1.56 μm, with an average value of $0.76 \pm 0.02$ μm; the hyphal diameter of those from invertebrates intestines varied from 0.18 to 1.06 μm, with an average value of $0.46 \pm 0.01$ μm. A true mycelium was not revealed in the excrement of the animals.

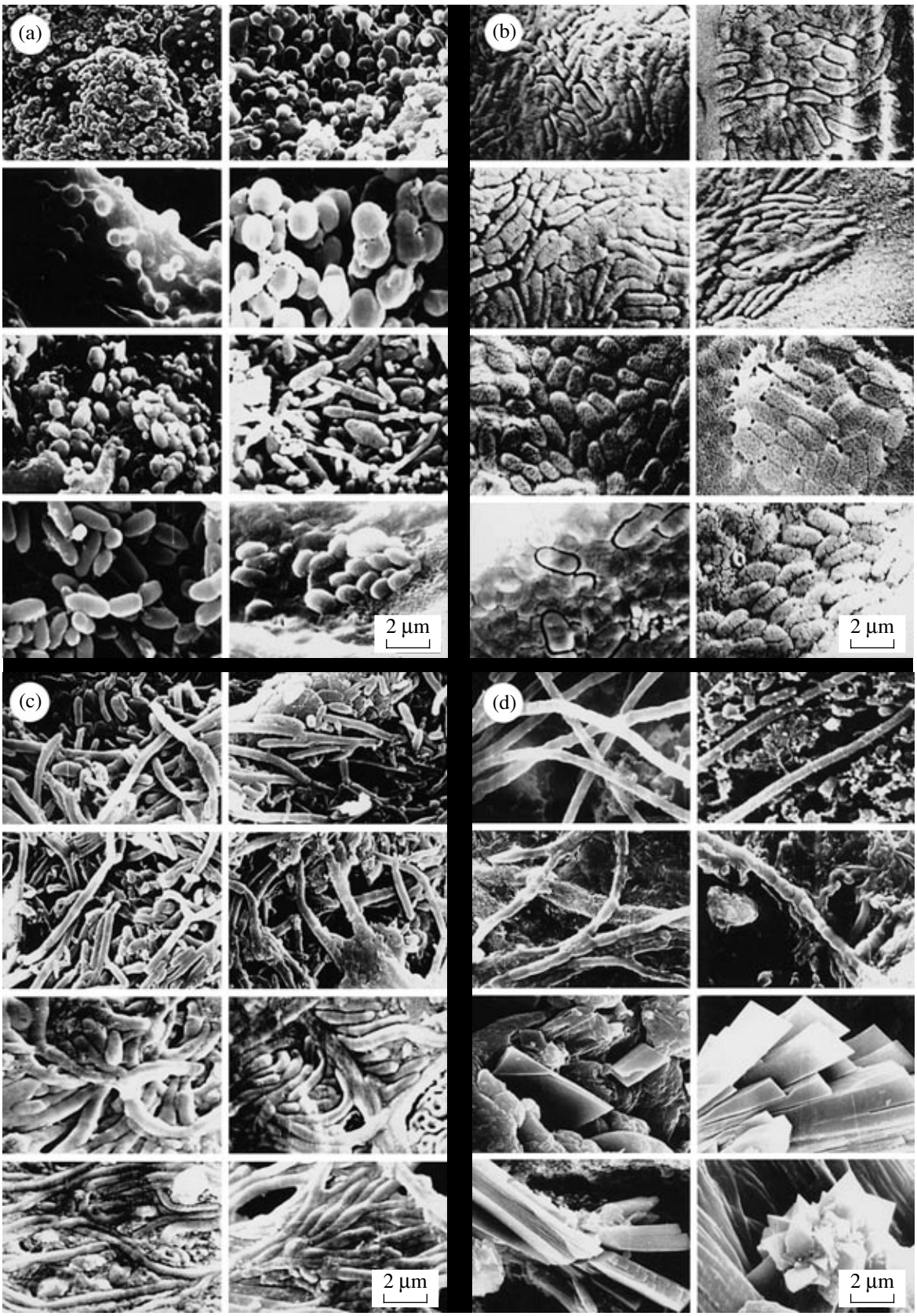


**Fig. 1.** Morphological diversity of bacteria associated with soil millipedes: (a) coccoid and oval cells in the intestine; (b) rod-shaped cells in the excrement; (c) branched rod-shaped cells, forming rudimentary mycelium, in the intestine; and (d) filamentous hyphae before and after fragmentation into short rods in the intestine and crystalline new formations in the excrement.

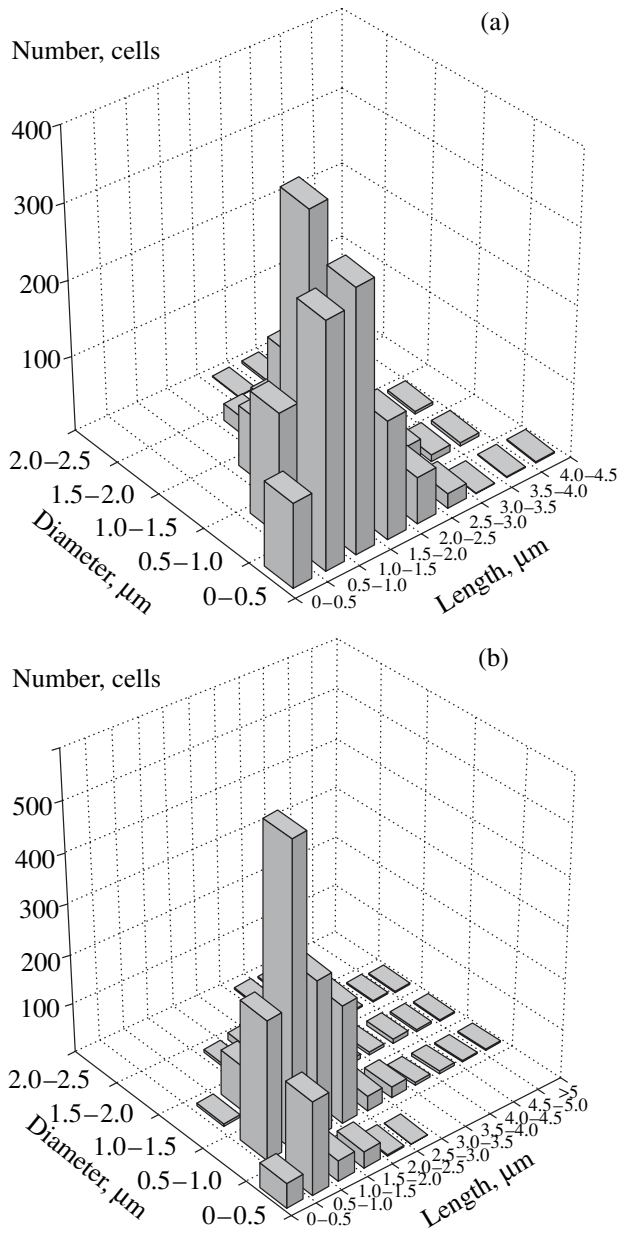


**Fig. 2.** Histogram of distribution by size of bacterial cells associated with millipedes (a) and functioning directly in soil (b).

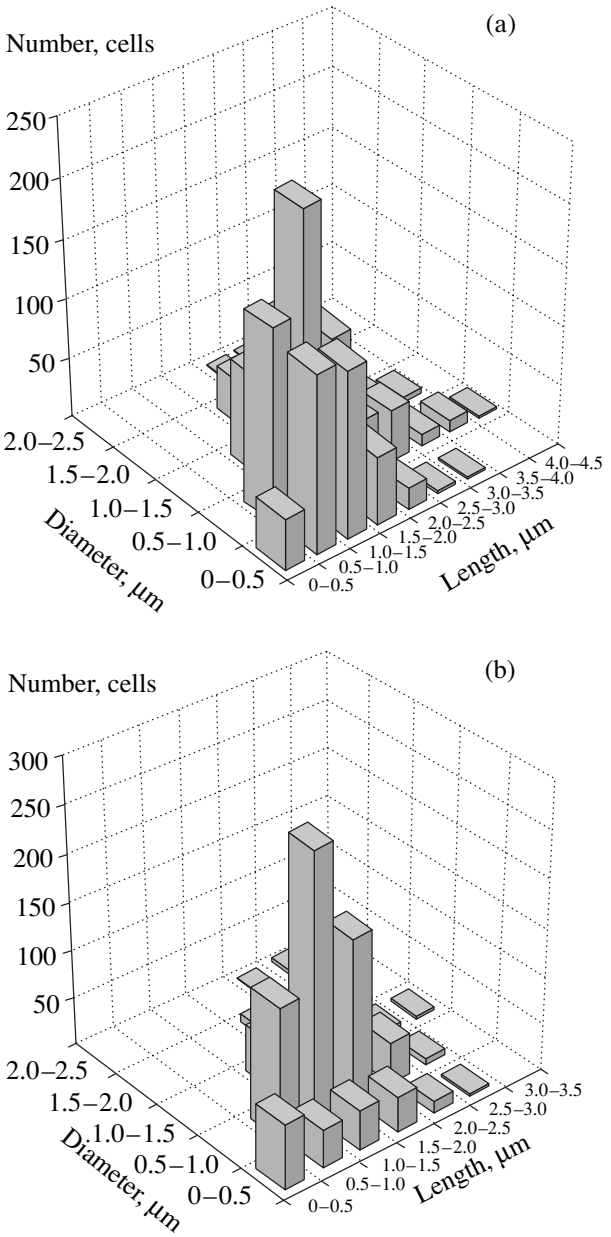


**Fig. 3.** Histogram of distribution by size of bacterial cells functionally associated with the intestine (a) and the excrement of millipedes (b).

In the diagrams (Fig. 5), the biometric characteristics of the subsets of all the bacterial cells studied were compared. The averaged bacterial cell associated with millipedes, including their intestinal and excremental bacteria, is evidently a slightly elongated rod 0.65 μm in diameter and 1.36 μm in length. Intestinal and excrement cells slightly differ in size. In the intestine, bacterial cells are longer by an average of 7% and thinner by an average of 6% than in excrement. Significant differences between the average-volume value between intestinal and excremental cells were not found. However, the ave rage cell sizes of millipede-associated bacteria are significantly smaller in diameter (Fig. 5a), length (Fig. 5b), and volume (Fig. 5c) than those of the bacteria directly functioning in soil. Taking the average size of soil microorganisms to be 100%, a decrease in diameter was 26 and 19%, in length it was 10 and 16%, and in volume was 33 and 42%, respectively. These differences are significant with a high degree of probability.

## DISCUSSION

The results of the biometric analysis of the microbial population of the intestinal tract of invertebrates may be useful for understanding the specific features of this specific habitat. The main difference between the

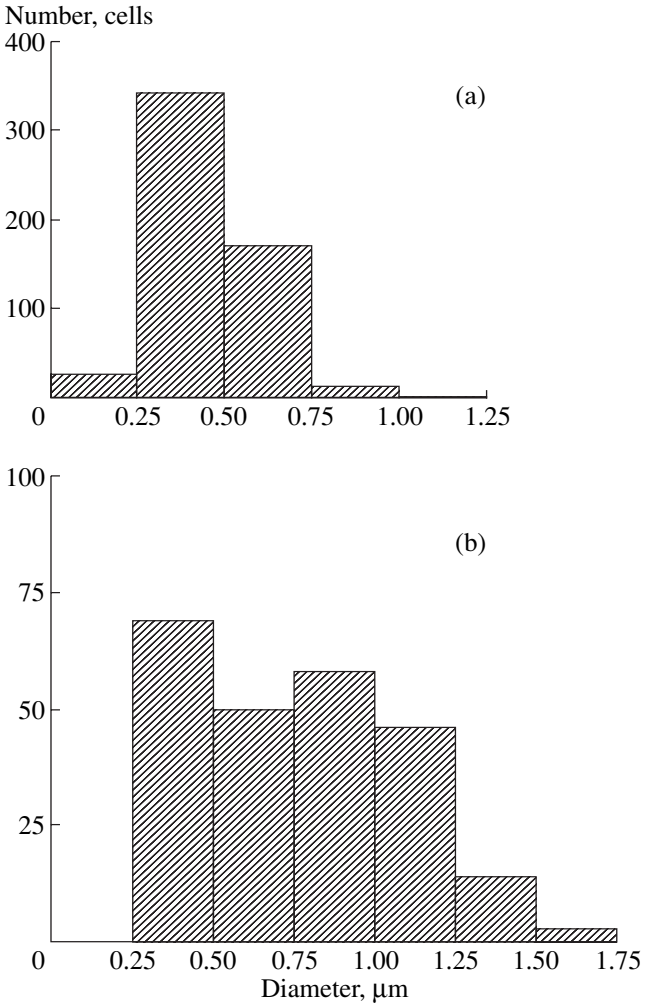


**Fig. 4.** Histogram of distribution by the size of the hyphal diameter of the actinomycetes revealed in the millipede intestine (a) and directly in soil (b).

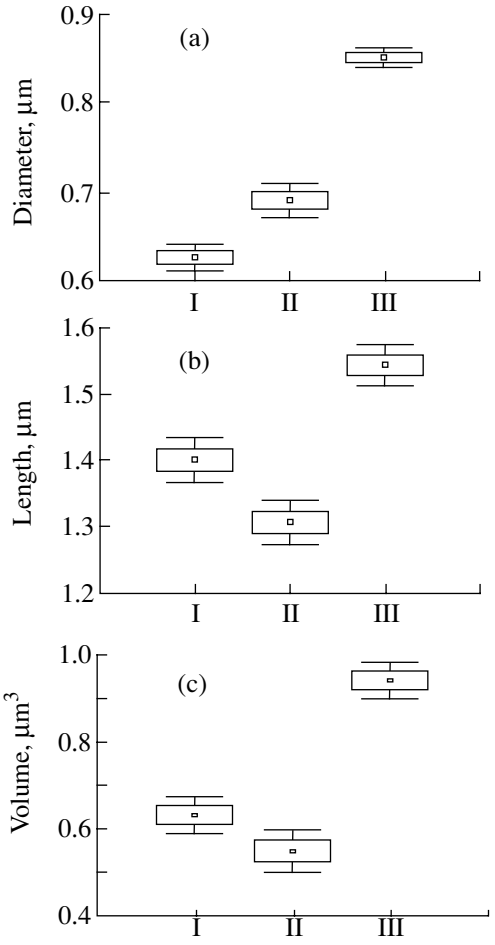


**Fig. 5.** The average value of (a) the diameter, (b) the length, and (c) the volume of bacterial cells (I) associated with the intestine of millipedes, (II) their excrement, and (III) functioning directly in soil. Designations: ⊥, ±1.96 standard error; ▭, ±1.00 standard error; □, average value.

state of the microbes associated with the intestinal tract of invertebrates and of soil microbes proper consists in that the former are always present in the form of vegetative cells (Fig. 1), whereas part of the bacteria in soil are present as spores [8]. The transition to a resting state in microorganisms is directly connected with the synthesis of low molecular weight autoregulators limiting their growth [9]. A comparison of these data leads to the conclusion that the intestinal tract of invertebrates acts as a sort of a flow system, where microorganisms are more prone to vegetative growth, without the accumulation of high concentrations of growth-inhibiting metabolites.

The general biological rule, according to which the metabolic rate is inversely proportional to body size is believed to be true at all the levels of biological complexity. Therefore, a small size gives bacteria a biological advantage in many situations, when they compete for nutrients with larger organisms [10]. Since cells differ greatly in their shape, cell volume may serve as a suitable parameter for comparing their magnitudes. The mean value of the volume of the cells inhabiting the millipede intestinal tract is less than the volume of those inhabiting soil, by 36% (Fig. 4). The finding of these differences confirms the previously formulated proposition that increased bacterial metabolic activity is not only accompanied by concentration changes, but also by allometric changes in a microbial community. In loci with higher microbial activity, as in the intestine of invertebrates in our case, not only do bacteria have denser populations, but the cells are also smaller in size [8].

In order to identify microorganisms by size and morphology, we compared our results to the literature data on the taxonomic composition of millipede intestinal bacteria, revealed by the inoculation method. The taxa earlier revealed were assigned to one or another morphological group using the generic morphological characteristics of Bergey's Manual of Determinative Bacteriology [12]. According to the results of this study, cocci account for about 20% of the microbial population of millipede intestines. According to the literature data, this group, in the order of increasing cell

diameter, may be represented by bacteria of the genera *Micrococcus* [13, 14], *Rhodococcus* [15], *Sarcina* [13, 14], and *Azotobacter* [15]. Nonsporeforming rods with a diameter not exceeding 1 μm and a length of less than 2 μm predominate in the intestinal community of millipedes. The genera *Alcaligenes* [15], *Enterobacter, Erwinia* [13–15], *Vibrio*, and *Flavobacterium* [15] may represent the group of smaller rods. The group of medium-sized rods may be represented by *Escherichia* [15], *Pseudomonas* [14, 15], *Klebsiella*, and *Mycobacterium* [14, 15]. The group of cells with a more elongated form may be represented by bacteria of the genera *Beijerinckia* [13], *Salmonella, Corynebacterium* [15], and *Cytophaga* [13]. Since the portion of this morphological type of bacteria is more than 50%, these organisms may be assigned to the typical representatives of the intestinal microflora of soil millipedes.

Organisms with mycelial cell organization should be considered among the typical bacteria of the millipede intestine. These are both the actinomycetes proper and the bacteria assigned to the actinomycetes line, but differing from them in either the absence of a true mycelium or its presence only at the early stages of development, with subsequent fragmentation into rod-shaped elements. The representatives of the genera *Micromonospora* and *Promicromonospora* [13, 15–17] may form a group with thinner hyphae, while the organisms of the genera *Streptomyces, Streptoverticillium, Streptosporangium* [16], and *Nocardia* [13] could represent a group with thicker hyphae (Fig. 4). Finally, the inoculation of medium with the millipede intestinal material frequently revealed bacteria attributed to genus *Bacillus* [13–15, 18]. Microscopy revealed the portion of these comparatively large rods to account for no more than 10% (Fig. 3).

As for a taxonomic position of the microorganisms inhabiting the millipede excrement, we succeeded in finding only fragmentary evidence in the literature. The presence of bacteria of the genus *Promicromonospora* is noted in some publications [16, 18]. However, in our investigations, not once were the mycelial forms of bacteria revealed in the excrement. Therefore, we suggest that these organisms cannot be considered typical for this habitat, and may be soundly assigned to transitory ones. A biometric analysis of the microbial population of the intestine and excrement shows their close similarity (Fig. 3). A decline in the portion the so-called thin rod groups, in the excremental microbial community (the lower horizontal row in Fig. 3b), should be considered the main distinction. The bacteria of the genera *Corynebacterium, Cytophaga, Klebsiella*, and *Mycobacterium* may have such sizes. Based on the aforesaid, it can be stated that the results obtained by means of microscopy and the inoculation method do not contradict each other.

It is interesting to compare the results of the in situ biometric analysis of the microbial population of the invertebrate intestinal tracts, with the size ranges that are indicated as characteristics of the generic taxa of bacteria grown in nutrient media in the laboratory. Thus, for example, in the mycelial microorganism groups, ones that are commonly isolated from the intestine are representatives of the following three genera: *Micromonospora*, in which hyphae about 0.5 μm in diameter are inherent according to *Bergey's Manual of Systematic Biology* [11]; *Streptomyces* whose hyphal sizes vary between 0.5 and 2.0 μm; and, finally, *Streptoverticillium* actinomycetes whose hyphal diameter is in the range of 1.0–2.0 μm. The results of measuring actinomycete hyphae obtained in our work (Fig. 4) indicate that all the hyphae revealed in the intestine fit in the 0.25–1.0 μm range, which corresponds to the lower range values typical for cultures grown in nutrient media. The same conclusion follows from comparing the size characteristics for other morphological groups, small cocci, thin short or elongated rods.

Thus, this experimental study, exemplified by the bacterial community of the millipede intestine, showed the bacteria of the intestinal tract of soil invertebrates to have the following features: (1) they are present in this habitat at high densities; (2) they are predominantly present in the form of vegetative cells; (3) they are significantly smaller in size than bacteria functioning in soil; (4) their sizes are close to the lower limits of the range typical of cultures grown in laboratory nutrient media. These characteristics suggest that a bacterial community of a digestive tract differs from a typical soil community not only in composition, but also in a higher level of physiological activity. In summarizing, we would like to stress the fact that new experimental evidence was obtained in this work, in favor of the concept of a medium-forming role of soil invertebrates in the saprotrophic process.

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