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EXPERIMENTAL ARTICLES

Reaction of Microorganisms to the Digestive Fluid of Earthworms

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Abstract—The reaction of soil bacteria and fungi to the digestive fluid of the earthworm *Aporrectodea caliginosa* was studied. The fluid was obtained by centrifugation of the native enzymes of the digestive tract. The inhibition of growth of certain bacteria, spores, and fungal hyphae under the effect of extracts from the anterior and middle sections of the digestive tract of *A. caliginosa* was discovered for the first time. In bacteria, microcolony formation was inhibited as early as 20–30 s after the application of the gut extracts, which may indicate the nonenzymatic nature of the effect. The digestive fluid exhibited the same microbicidal activity whether the earthworms were feeding on soil or sterile sand. This indicates that the microbicidal agents are formed within the earthworm's body, rather than by soil microorganisms. The effect of the digestive fluid from the anterior and middle divisions is selective in relation to different microorganisms. Of 42 strains of soil bacteria, seven were susceptible to the microbicidal action of the fluid (*Alcaligenes faecalis* 345-1, *Microbacterium* sp. 423-1, *Arthrobacter* sp. 430-1, *Bacillus megaterium* 401-1, *B. megaterium* 413-1, *Kluyvera ascorbata* 301-1, *Pseudomonas reactans* 387-2). The remaining bacteria did not die in the digestive fluid. Of 13 micromycetes, the digestive fluid inhibited spore germination in *Aspergillus terreus* and *Paecilomyces lilacinus* and the growth of hyphae in *Trichoderma harzianum* and *Penicillium decumbens.* The digestive fluid stimulated spore germination in *Alternaria alternata* and the growth of hyphae in *Penicillium chrysogenum.* The reaction of the remaining micromycetes was neutral. The gut fluid from the posterior division of the abdominal tract did not possess microbicidal activity. No relation was found between the reaction of microorganisms to the effects of the digestive fluid and the taxonomic position of the microorganisms. The effects revealed are similar to those shown earlier for millipedes and wood lice in the following parameters: quick action of the digestive fluid on microorganisms, and the selectivity of the action on microorganisms revealed at the strain level. The selective effect of the digestive gut fluid of the earthworms on soil microorganisms is important for animal feeding, maintaining the homeostasis of the gut microbial community, and the formation of microbial communities in soils.

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Key words: bacteria, fungi, earthworms, digestive fluid, microbicidal activity.

Interactions between earthworms and microorganisms are important for soil processes such as decomposition and transformation of plant residue, humus formation, and the formation of the pool of nutrient elements and microbial communities. The wide spectrum of these interactions makes it possible to speak about a close relationship between earthworms and microorganisms. Fungi and bacteria are assumed to be the main source of food for earthworms [1, 2]. While feeding, earthworms regulate the growth of soil microorganisms by eating some microbial populations and providing favorable conditions for the growth of others in their digestive tract and in casts [3–13]. The rate of food transit along the digestive tract of earthworms is very high (completed within several hours). Therefore, it appears that earthworms quickly kill and digest the soil microorganisms consumed. It has previously been shown that the digestion of microorganisms in the gut of invertebrates (millipedes and wood lice) begins with the quick death of certain microbial cells caused by specific killing agents of nonprotein nature [14–16]. No similar studies for earthworms have been conducted. However, it is likely that selective feeding of invertebrates on microorganisms is one of the fundamental principles of zoomicrobial interactions. Moreover, the selection of microorganisms in the digestive tract of earthworms

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may be an important factor of the formation of microbial communities in soil.

The goal of the present work was to characterize the effects of the digestive fluid from different divisions of the digestive tract of the earthworm *Aporrectodea caliginosa* on soil bacteria and fungi.

MATERIALS AND METHODS

Soil. Cultivated soddy-podzolic soil under leguminous–cereal vegetation being used in a multiyear experiment of the Agrochemistry Department, Moscow State University (Chashnikovo Soil–Ecological Center, Moscow State University, Moscow oblast) was used. The soil content of total carbon was 1.72%; that of nitrogen, 0.13%; and the pH of the aqueous extract was 5.7. In 1989, lime was applied in doses sufficient to neutralize the hydrolytic acidity. During the 1990–1993 period, the soil received 40 t/ha of organic fertilizes (dung) and mineral fertilizers $N_{100}P_{500}K_{500}$ per year. In 1994–1999, the soil received an average of $N_{100}P_{40}K_{100}$ per year.

Earthworms. The earthworm *Aporrectodea caliginosa* inhabiting the 0–20-cm horizon of this soil was used in the work. The earthworms were collected in autumn 2003 and maintained at 12–15°C in containers with soil.

Microorganisms. Bacteria and fungi were isolated from the soil by plating on R2A agar and Czapek acidified agarized medium, respectively.

Preparation of the cultures for PCR amplification. The bacterial and fungal isolates from soil were grown in 50 μl of Czapek liquid medium in a 96-well microtiter plate (NUNC™, Denmark) at 30°C for four days. The liquid culture from every well was then collected, and the biomass was washed off once with 100 μl of PBS (phosphate-buffered saline) solution (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 2 mM KH_2PO_4). The fungal biomass was subjected to the lytic procedure including the following steps: (1) incubation for 2 h with Y1 buffer (sorbitol, 1 M; EDTA, 0.1 M; lyticase, 100 U/ml) (RNA/DNA Mini Kit; QIAGEN, Germany) and (2) after removal of Y1 buffer, incubation with 100 μl of PCR lysis solution A (67 mM Tris-HCl (pH 8.8); 16 mM $(NH_4)_2SO_4$; 5 μM β-mercaptoethanol; 6.7 mM $MgCl_2$; 6.7 mM EDTA (pH 8.0); 1.7 μl SDS (sodium dodecyl sulfate); 50 μg/ml proteinase K) [17] for 4–8 h at 55°C. Proteinase K deactivation was carried out by heating the lysate at 80°C for 10 min. The bacteria were lysed in PCR lysis solution A immediately after washing off in PBS.

PCR amplification of bacterial 16S rDNA and fungal 28S rDNA (the D1/D2 domain). The reaction was performed in the volume of 20 μl. The reaction mixture was composed according to the manufacturer's (QIAGEN GmbH, Germany) recommendations: deionized water, 9 μl; Q solution, 4 μl; 20 mM dNTPs solution (pH 8.0), 4 μl; PCR-buffer ×10 (contains 15 mM $MgCl_2$), 2.0 μl; 1.0 U Taq DNA-polymerase; 0.12 nmol of each primer (NL-1 (upstream) [5'-GCATATCAATAAGCGGAGGAAAA-3']) and NL-4 (downstream) [5'-GGTCCGTGTTTCAAGACGG-3']) and 2 μl lysate (for fungi); and 2 universal primers F27 (upstream) [5'-AGAGTTTGATCMTGGCTCAG-3'] and R1492 (downstream) [5'-TACGGYTACCTTGTTACGACTT-3'] and 1 μl lysate (for bacteria). The amplification conditions were as follows: initial denaturation at 95°C for 2 min followed by 30 cycles consisting of 1 min denaturation at 95°C, 1 min annealing at 50°C, and 1.5 min amplification at 72°C; and final annealing at 72°C for 10 min. The reaction was performed in the Eppendorf 3541 thermocycler (Germany). The amplification success (the size of the PCR product was about 680 nucleotides (fungi) and 1400 nucleotides (bacteria)) was tested with 2% agarose gel electrophoresis in a 96-well format (Invitrogen, Germany).

Amplicon sequencing and analysis of the data obtained. Before sequencing, the PCR products were purified using the MinElute 96 UF PCR purification unit (QIAGEN, Germany). Unidirectional sequencing was carried out using the NL-4 and R1492 primers in Eppendorf 5341 (Germany) according to the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, United States) protocol. The ready-for-use reaction mixture consisted of Ready Reaction Premix ×2.5, 4 μl; BigDye Sequencing Buffer ×5.0, 2 μl; primer, 10 pcmol; PCR product, 60 ng; deionized water, 12 μl. The amplification conditions: 25 cycles consisting of 20 s at 96°C, 20 s at 50°C, and 4 min at 60°C. The data obtained were processed using the BioEdit program; the identification was carried out using the GenBank BLAST alignment software (http://www.ncbi.nlm.nih.gov/BLAST/) [18].

Identification of fungi by cultural and morphological characteristics. The identification of microscopic fungi was carried out using the manuals relevant for a specific taxonomic group. Thirteen strains of the following species were used in this work: *Alternaria alternata* 1C321, *Aspergillus versicolor* 1K213, *A. terreus* 3P422, *Gliocladium catenulatum* 2P723, *G. roseum* 2P522, *Fusarium oxysporum* 2P433, sterile mycelium 7K521, *Paecilomyces lilacinus* 2P513, *Penicillium aurantiogriseum* 5P623, *P. chrysogenum* 4E232, *P. decumbens* 3P211, and *Trichoderma harzianum* 1E211. The strain represented by sterile mycelium and regularly revealed in the digestive tract of the earthworm *A. caliginosa* [19] was identified as *Bjerkandera adusta* 7K521 by molecular–genetic methods.

Cultivation of microorganisms for biological testing. Bacteria and micromycetes isolated from the soddy-podzolic soil, as well as the yeast *Saccharomyces cerevisiae*, were used as test objects. The bacterial cultures were grown in penicillin vials in the liquid R2A medium of the following composition: yeast extract, 0.5 g; peptone, 0.5 g; casamino acids, 0.5 g; glucose, 0.5 g; starch, 0.5 g; sodium pyruvate, 0.3 g;

disodium phosphate, 0.3 g; magnesium sulfate, 0.05 g; distilled water, 1 l. The incubation was performed on a shaker at 180 rpm for 24 h at 18–20°C. The cells were washed off the medium by double centrifugation at 8000 *g* in sterile tap water. Micromycetes were grown on wort agar for 7–10 days. The yeast cultures were grown in liquid wort for 18–20 h. The growth temperature was 18–20°C.

Preparation of the digestive fluid. To determine the survivability of bacteria and fungi in the digestive fluid of the earthworms, the animals were preliminarily kept either in soil or in sand sterilized by autoclaving. This procedure made it possible to determine whether or not the substrate entering the digestive tract influences the activity of the digestive fluid. To isolate the digestive fluid, the earthworms were killed by immersing them in boiling water for 1 s. The earthworm was then placed on the surface of a freezing table (Peltier element) where it was cooled down to –16°C within 1–2 min. The earthworm was then dissected, and at the moment of thawing out, the gut and/or its contents were removed from the anterior and middle (immediately behind the clitellum) and posterior divisions, care being taken not to allow repeated freezing of the material. The mass removed was centrifuged at 8000 *g* for 5 min for the contents to precipitate, including the bacteria and fungi. The undiluted supernatant fluid was tested for sterility by inoculation of R2A agar and subsequent incubation at 20°C for seven days. No bacteria or fungi were revealed in the supernatant of the digestive fluid. A total of 300 earthworms were dissected. Thus, we obtained the digestive fluid from the anterior, middle, and posterior divisions of the gut, which were either emptied of soil or filled with it. Before the beginning of testing, the gut fluid was stored at –18°C.

Determination of the survivability of bacteria and fungi. The viability of bacteria was determined by counting the colony-forming units (CFU) when the agar films were inoculated with microbial suspensions. The microscope slides were freed from fat by being ignited over a burner, placed into Petri dishes, and overlayed with agarized R2A medium so that the layer thickness was 1.5 to 2 mm. The slide with the solidified medium was transferred onto sterile filter paper in a petri dish. Bacterial suspension (1 μl, 10^3 and 10^4 cells/ml) was applied to the agarized medium. The digestive fluid (2 μl) was added to the dried suspension drops. The digestive fluid was imbibed by the agar in 20–30 s. In the control, sterile tap water was added to the suspension. The dishes were incubated in a desiccator at 18–20°C for 24 h. The number of microcolonies was determined under a light microscope at ×32 and ×125. To calculate the error when applying the suspension, aliquots (1 μl) from the corresponding dilution were applied to the agarized medium in 12 repetitions. The mean error by the number of colonies did not exceed 7% (e.g., 161 ± 10/ml).

The reaction of fungi to the effects of the digestive fluid of the earthworms was determined in a similar way by applying 1 μl of the spore suspension to the slides covered with R2A agar. The digestive fluid (2 μl) was applied to drops of suspension after they had dried. The slides were incubated in a desiccator at 18–20°C for 24 h. The germinating spores were counted under a light microscope at ×400. When assessing the level of germination, no less than 100 spores were taken into account (the density of applying the spores on the slide was 4–15 in the field of vision at ×400).

In another experimental variant, the fungal mycelium was ground by agitation in a vortex. The suspension of hyphal fragments was mixed with digestive fluid in a 1 : 1 ratio. Petri dishes with agarized Czapek medium were inoculated with the mixture 1–2 min, 2 h, and 24 h after mixing with the fluid. The incubation was carried out at 18–20°C for 24 h. The number of colonies grown was then determined.

Microscopy. The bacterial microcolony number, the germination of the micromycete spores, and the morphological changes in the cells of the yeast *Saccharomyces cerevisiae* were assessed when influenced by the digestive fluid. The yeast cells were separated by centrifugation at 2000 *g* for 5 min and resuspended in tap water; the suspension was applied on the microscope slide; the digestive fluid was added; and microphotographs were taken 5 min later.

RESULTS

The isolation and identification of test microorganisms. To test the reaction of microorganisms to the effect of the digestive fluid of the earthworms, 42 bacterial strains and 13 micromycete strains were isolated from the soddy-podzolic soil. According to the data of PCR amplification of bacterial 16S rDNA and fungal 28S rDNA (D1/D2 domain), the microorganisms were assigned to the following taxa (Table 1).

Reaction of the bacteria to the effect of the digestive fluid of the earthworms. The agar film method allows the microcolonies formed by several dozens of cells to be visualized under the light microscope and the capacity of microorganisms for growth to be studied when working with small volumes of cell suspensions. This is important, since the volume of the digestive fluid from one earthworm constitutes several μl. The effect of the digestive fluid of the earthworms on microorganisms was assessed by the development of microcolonies and their number.

To determine the exposure time causing bacterial death, the cell suspension of *Kluyvera ascorbata* 303-1 was mixed with the digestive fluid and plated on the agar films 20–30 s; 1, 1.5, 3, 6, 9, 15, and 30 min; and 1, 1.5, and 2 h after mixing. The number of the microcolonies formed was determined after 24 h. No bacteria grew even after 20–30 s of exposure to the digestive fluid.

Table 1. The microorganisms studied in the work

Class	Microorganisms
	Bacteria
Alphaproteobacteria	*Aminobacter* sp. 411-1, 411-2, 408-1, 408-2 (close to *A. aminovorans* [AF250404]* and to *A. niigataensis* [AJ011761]) *Brevundimonas diminuta* 384-1 [X87274] *Sphingopyxis witflariensi* 397-1 [AJ416410]
Betaproteobacteria	*Alcaligenes faecalis* 345-1 (the closest *A. faecalis* subsp. *parafaecalis* [AJ242986]) *Bordetella* sp. 341-1 (the closest *B. petri* [AJ870969]) *Delftia acidovorans* 335-1 [AF538930]
Gammaproteobacteria	*Kluyvera ascorbata* 303-1 [AF008579] *Pseudomonas proteolytica* 599 [AJ537603] *P. putida* 348-1 [D37923] *P. reactans* 383-1, 387-2, 394-2, 400-2 [AF255337] *Pseudomonas* sp. KL28 (309-2) [AY324319] *Pseudomonas* sp. 329-1 (similar to *P. brassicacearum* [AJ292381] and *P. migulae* [AF074383]) *Pseudomonas* sp. 387-1 (sp. Dp2 [AF288736]) *Pseudomonas* sp. 399-2 (close to MG1 [AF326378], cb3 [AF351240], R1enr (sp. M29) [AY263479]) *Pseudomonas* sp. 607-1 (the closes *P. poae* [AJ492829] and to *P. synxantha* [AF267911])
Actinobacteria	*Artrobacter oxydans* 304-2 [X83408] *Agromyces cerinus* 347-1 [X77448] *Arthrobacter globiformis* 333-1[AB098573] *Arthrobacter* sp. 392-1, 430-1 [D84573] *Kocuria palustris* 405-2, 416-2 [Y16263] *Microbacterium* sp. 423-1 *Nocardioides* sp. 410-1 (close to *N. fulvus*) [AF537327]) *Rhodococcus opacus* 404-2 [X80631] *Streptomyces* sp. 406-2 (close to *S. griseus*) *Streptomyces* sp. ASSF22 389-1 [AF012738]
Bacilli	*Bacillus licheniformis* 414-2 [AF387514] *B. mojavensis* 317-1 [AY030339] *B. megaterium* 401-1, 413-1, 413-2 [AY030338] *B. subtilis* 385-2, 386-2 [AY030331] *Paenibacillus* sp. DSM 1352, 412-1, 412-2 [AJ345017]
	Fungi
Ascomycetes	*Alternaria alternata* 1C321 [137]** *Aspergillus versicolor* 1K213 [561] *A. terreus* 3P422 [368] *Gliocladium catenulatum* 2P723 [476] *G. roseum* 2P522[237] *Fusarium oxysporum* 2P433 [512] *Paecilomyces lilacinus* 2P513 [984] *Penicillium aurantiogriseum* 5P623 [725] *P. chrysogenum* 4E232 [314] *P. decumbens* 3P211 [611] *Trichoderma harzianum* 1E211 [293]
Basidiomycetes	*Bjerkandera adusta* 7K521 [897]

* Reference to the source in the Internet: GenBank NCBI.

** www.indexfungorum.org.

Table 2. Reaction of the bacteria isolated from the soddy-podzolic soil to the effect of the digestive fluid of the anterior and middle divisions of the earthworm *Aporrectodea caliginosa* (only the strains with 99–100% similarity to the corresponding library species were used)

Bacterium	Number, CFU/μl	
	control	experiment
Microocolony formation is inhibited		
Alcaligenes faecalis 345-1*	1500	23
Microbacterium sp. 423-1	over 5000	120
Arthrobacter sp. 430-1	520	57
Bacillus megaterium 401-1	36	0
B. megaterium 413-1	78	2
Kluyvera ascorbata 303-1	146	18
Pseudomonas reactans 387-2	137	4
No effect		
Agromyces cerinus 347-1	55	50
Aminobacter sp. 411-1	64	73
Aminobacter sp. 411-2	213	138
Aminobacter sp. 408-1	62	46
Aminobacter sp. 408-2	56	58
Arthrobacter globiformis 333-1	468	439
A. oxydans 304-2	237	219
Arthrobacter sp. 392-1	156	145
Bacillus licheniformis 414-2	96	112
B. megaterium 413-2	108	96
B. mojavensis 317-1	74	58
B. subtilis 385-2	238	218
B. subtilis 386-2	328	342
Bordetella sp. 341-1	175	162
Brevundimonas diminuta 384-1	139	120
Delftia acidovorans 335-1	268	240
Kocuria palustris 405-2	142	126
K. palustris 416-2	128	104
Nocardioides sp. 410-1	461	436
Paenibacillus sp. 412-1	215	232
Paenibacillus sp. 412-2	256	183
Pseudomonas proteolytica 599	833	741
P. putida 348-1	550	450
P. reactans 383-1	76	91
P. reactans 394-2	224	185
P. reactans 400-2	187	149
Pseudomonas sp. KL28 (309-2)	98	87
Pseudomonas sp. 329-1	600	560
Pseudomonas sp. 387-1	60	53
Pseudomonas sp. 399-2	200	168
Pseudomonas sp. 607-1	247	219
Rhodococcus opacus 404-2	46	68
Sphingopyxis witflariensis 397-1	320	120
Streptomyces sp. 389-1	171	153
Streptomyces sp. 406-2	88	72

* The strain numbers of the collection at the Department of Soil Biology, Moscow State University, are given.

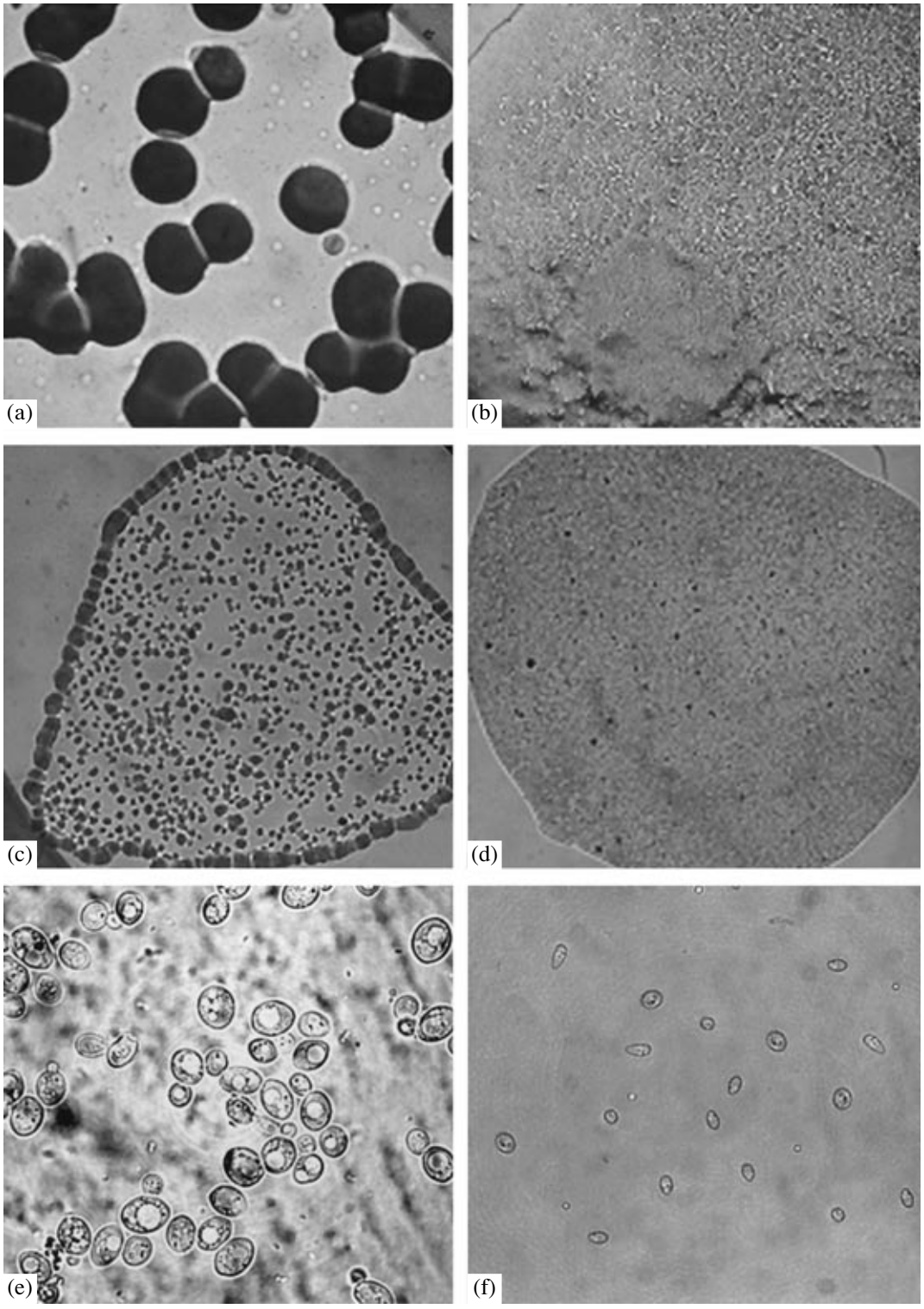
Table 3. Reaction of the bacteria isolated from the soddy-podzolic soil to the effect of the digestive fluid from the posterior division of the earthworm *Aporrectodea caliginosa* (only the strains with 99–100% similarity to the corresponding library species were used)

Bacterium	Number, CFU/μl	
	control	experiment
No effect		
Kluyvera ascorbata 303-1	146	158
Bordetella sp. 341-1	175	140
Delftia acidovorans 335-1	268	248
Pseudomonas sp. 309-2	98	106
Arthrobacter globiformis 333-1	451	468
Arthrobacter sp. 430-1	512	520
Arthrobacter sp. 392-1	162	156
Stimulation of microcolony formation		
Pseudomonas putida 348-1	550	>1000
Pseudomonas sp. 329-1	560	>1000
Pseudomonas sp. 387-1	60	111

On exposure to the digestive fluid from the anterior and middle gut divisions, several bacteria, e.g., *Bacillus megaterium* 401-1 and *Alcaligenes faecalis* 345-1 virtually lost the capacity for microcolony formation (Figs. 1a–1d). However, not all the bacteria tested died on exposure to the digestive fluid of the earthworms. A total of seven highly sensitive strains were revealed. Their number decreased tenfold or more after short-term (20–30 s) contact with the digestive fluid. The cells of many strains (35) did not die (Table 2).

The fluid obtained from the posterior division of the digestive tract did not exert a bactericidal effect but, on the contrary, stimulated the formation of microcolonies in three of the bacterial strains tested: *Pseudomonas putida* and *Pseudomonas* sp. (two strains) (Table 3).

To reveal the source of the origin of the microbicidal activity in the gut (the animal's own activity or activity connected with the soil absorbed), the earthworms were kept in soil and in sterile sand. The comparative analysis of the biological activity of the digestive fluids of the earthworms kept in sterile sand and in soil did not reveal significant differences in their effects on the test microorganisms. The effects were quantitatively simi-



Reaction of bacteria and yeasts to the effect of the gut fluid from the anterior and middle divisions of the earthworm *Aporrectodea caliginosa.* Microcolonies of *Bacillus megaterium* 401-1: (a) control, ×84; (b) experiment, ×84. Microcolonies of *Alcaligenes faecalis* 345-1: (c) control, ×21; (d) experiment, ×21. Cells of *Saccharomyces cerevisiae*: (e) control, ×670; (f) 5 min after exposure to the gut fluid, ×670.

Table 4. Comparative analysis of the effect on the bacteria of the digestive fluid from the anterior and middle (AMD) and posterior (PD) divisions of the digestive tract of the earthworm *Aporrectodea caliginosa* kept in soil (soil) and sterile sand (sand)

Bacteria	Number, CFU/μl				
	control	sand		soil	
		MD	PD	MD	PD
Alcaligenes sp. 345-1	>1000	18	>1000	23	>1000
Arthrobacter globiformis 333-1	468	445	453	439	451
Arthrobacter sp. 392-1	156	145	172	143	162
Arthrobacter sp. 430-1	520	48	506	57	512
Bordetella sp. 341-1	175	154	210	162	140
Delftia acidovorans 335-1	268	254	258	240	248
Kluyvera ascorbata 303-1	146	21	153	18	158
Pseudomonas putida 348-1	550	467	>1000	450	>1000
Pseudomonas sp. 309-2	98	92	102	87	106
Pseudomonas sp. 329-1	560	960	>1000	>1000	>1000
Pseudomonas sp. 387-1	60	65	107	53	111
Pseudomonas sp. 399-2	200	176	115	168	159

Table 5. Reaction of the microscopic fungus spores to the effect of the digestive fluid of the earthworm *Aporrectodea caliginosa*

Species/strain	Spore germination, %	
	control	experiment
Microcolony formation is inhibited		
Aspergillus terreus 3P422	53.0 ± 4.8	7.0 ± 2.2
Paecilomyces lilacinus 2P513	78.7 ± 4.1	4.0 ± 1.3
No effect		
Aspergillus versicolor 1K213	83.1 ± 3.3	64.7 ± 3.6
Gliocladium catenulatum 2P723	64.6 ± 2.8	58.1 ± 2.6
G. roseum 2P522	75.0 ± 3.3	62.5 ± 2.0
Penicillium aurantiogriseum 5P623	58.9 ± 5.2	39.2 ± 3.0
Stimulation of microcolony formation		
Alternaria alternata 1S321	58.8 ± 4.0	75.0 ± 2.1

Note: ± is the standard deviation.

lar in relation to induction of bacterial death or growth stimulation, as well in relation to the absence of effects (Table 4).

Reaction of fungal spores. The inhibition of spore germination of the fungi of most of the species tested was observed even after short-term exposure to the digestive fluid from the anterior and middle divisions of the earthworm gut. The inhibitory effect of the digestive fluid varied from complete inhibition of spore germination in *Paecilomyces lilacinus* and *Aspergillus terreus* to a weaker inhibition of spore germination in *Gliocladium catenulatum, G. roseum* and slight stimulation of spore germination in *Alternaria alternata* (Table 5).

Reaction of fungal hyphae. The incubation of mycelial fragments of *Trichoderma harzianum* and *Penicillium decumbens* strains in the digestive fluid for 1–2 min led to their death. In the case of *Fusarium oxysporum, Paecilomyces lilacinus, Penicillium aurantiogriseum*, and *Gliocladium catenulatum*, radial growth rates of the fungal colonies did not change significantly after any period of exposure of their mycelium to the digestive fluid. *Bjerkandera adusta* showed an insignificant decrease in the radial growth rate, while in *Penicillium chrysogenum*, the radial growth rate increased when inoculated mycelial fragments were incubated in the gut fluid; this effect did not depend on the incubation time (Table 6).

Reaction of the *Saccharomyces cerevisiae* cells. Morphological changes to the cells of *S. cerevisiae* occurred after as little as 5 min of their exposure to digestive fluid from the anterior and middle divisions of the earthworm gut. The cells significantly shrank in volume and tapered; the vacuoles disappeared (Figs. 1e, 1f).

DISCUSSION

This is the first time we have assessed the effect of the gut fluid of the earthworm *Aporrectodea caliginosa* on the viability of microorganisms entering its digestive tract with soil [19, this work].

In a number of investigations using the plating and microscopy methods, a drastic increase in the number of bacteria was revealed from the anterior division of the earthworm gut to the posterior division and from the feed absorbed to their casts. On the contrary, the abundance of fungi declined [21–23]. The regulation of

Table 6. Reaction of the fungal mycelium to the effect of the digestive fluid of the earthworm *Aporrectodea caliginosa*

Species/strain	Radial growth rate, mm/h			
	control	exposure time		
		1–2 min	2 h	24 h
Fusarium oxysporum 2P433	0.10 ± 0.00	0.12 ± 0.00	0.10 ± 0.00	0.10 ± 0.01
Penicillium chrysogenum 4E232	0.09 ± 0.00	0.10 ± 0.00	0.11 ± 0.01	0.12 ± 0.01
Penicillium aurantiogriseum 5P623	0.12 ± 0.00	0.09 ± 0.01	0.12 ± 0.01	0.11 ± 0.01
Bjerkandera adusta 7K521	0.20 ± 0.02	0.16 ± 0.02	0.14 ± 0.01	0.15 ± 0.00
Gliocladium catenulatum 2P723	0.09 ± 0.02	0.09 ± 0.01	0.07 ± 0.01	0.08 ± 0.05
Paecilomyces lilacinus 2P513	0.11 ± 0.01	0.10 ± 0.02	0.13 ± 0.01	0.11 ± 0.00

Note: ± is the standard deviation.

reproduction and the stimulation of the growth of microorganisms in the gut of invertebrates may be accounted for by secretion or the formation of readily metabolized products, gut peristalsis, and a shift in pH and redox potential. Selective lysis of microbial cells may stimulate the growth and reproduction of the surviving microorganisms [24].

The degree of assimilation of microorganisms by soil invertebrates (for example, millipedes) is much higher than that of plant tissues; it can be as high as 80–95% of the total carbon of the microbial biomass consumed [16, 25]. Similar data for earthworms are absent. However, it seems very likely that due to the quick transit time of the feed substrate along the digestive tract of the earthworms (several hours), they should kill, digest, and effectively assimilate microorganisms contained in the soil.

The suppressing effect of the digestive fluid of the earthworms was first shown with protozoa [26]. Later, an antimicrobial killing activity against a series of bacterial, yeast, and fungal strains isolated from different habitats was revealed in the digestive extracts of the soil millipedes *Pachyiulus flavipes, Rossiulus kessleri*, and *Megaphyllum rossicum* and in the hepatopancreatic glands of the wood louse *Armadillidium vulgare.* In sensitive strains of bacteria, yeasts, and micromycetes, the death of more than 90% of the cells occurred during the first minutes of cell incubation in the gut fluid, as was demonstrated by plating and by microscopic observation of vacuole destruction (in fungi) [2, 15, 16].

Our data on the effects of the gut fluid of earthworms agree in many respects with the data obtained earlier. The primary effect is a high death rate. In the digestive fluid of earthworms, the death of bacterial and fungal cells occurs during the first minutes after their mixing with the fluid. Secondly, cell death is accompanied by drastic morphological changes. The reaction of the cells of the yeast *S. cerevisiae* to the effect of the gut fluid from the anterior and middle divisions of *Aporrectodea caliginosa* was similar to that observed earlier on exposure to the digestive fluid of the millipede *Pachyiulus flavipes* [15]. Only a few minutes after exposure, vacuole destruction and a sharp reduction in the size and shape of the yeast cells were seen under the light microscope (Figs. 1e, 1f).

Both gram-positive and gram-negative bacteria died in the digestive fluid of the earthworms (Table 2). Within each group, strain differences were noted in the reaction of tested bacteria. Along with the species which are highly sensitive to the action of the gut fluid, there exist species that are unaffected and species which show stimulation of growth (Table 3).

The gut medium appeared to be selective for fungi as well. Differences in the reaction of fungal spores and hyphae to the action of the digestive fluid of the earthworms were revealed. They varied from complete inhibition to stimulation of spore germination. A significant inhibition of the germination of *Aspergillus terreus* and *Paecilomyces lilacinus* spores and complete inhibition of the growth of colonies from the hyphae of *Trichoderma harzianum* and *Penicillium decumbens* were revealed. The inhibition of the *Mucor hiemalis* spore germination was first observed upon the action of the digestive fluid of *Lumbricus terrestris*, but only upon long-term (12-h) incubation in the gut fluid [11]. In our experiments, the inhibitory effect was revealed after 1–2-min contact of the spores with the native gut fluid. The stimulatory effect of the digestive fluid of the earthworms on *A. alternata* spore germination has been revealed by us for the first time. The activation of spore germination might be linked to partial destruction of the spore cell wall. This stimulating effect is consistent with this fungal species being commonly revealed in the gut of the earthworm *A. caliginosa* [27].

The digestive fluid of the earthworms feeding on soil and sterile sand showed the same microbicidal activity. This allows us to suggest that these microbicidal agents are formed in the earthworm body but not by the soil microorganisms entering their digestive tract. Earlier, it was shown that the killing activity of the millipede's digestive medium is not connected with the bacteria inhabiting the gut: partial sterilization of the gut with streptomycin did not lower the microbicidal activity [15].

Thus, the digestive medium of the earthworms is selective for the microorganisms gaining entrance into the gut with feed and soil. The experimentally observed selectivity of the reactions to the effect of the digestive fluid does not correlate with the taxonomic position of the tested microorganism.

It should be emphasized once again that the effect of rapid death (1–2-min contact) of microorganisms in the gut fluid of the earthworm *A. caliginosa* was only revealed in the medium of the anterior and middle divisions of the digestive tract. The fluid from the posterior division of the gut did not have any antimicrobial activity; on the contrary, the growth of microorganisms was stimulated by its action. Since the action of the gut fluid affects both gram-negative and gram-positive bacteria, as well as yeast cells, fungal spores and hyphae, the effects observed do not depend on the architecture of microbial cells, or the composition and structure of their cell walls. This leads us to suggest that the effect of the gut fluid of the earthworms on microorganisms is of a membranotropic character. It appears to be similar in its high action rate and selectivity at the strain level to the killing effect of the gut contents of millipedes and wood lice in relation to a number of bacteria and fungi [15, 16]. Several microorganisms were unaffected by the gut contents. Apparently, these could be classified as so-called transit microbes. There are also certain microorganisms whose growth is stimulated by the digestive tract contents. The mechanisms of these effects are unknown. Thus, microorganisms entering the digestive tract of earthworms meet with different fates. This is reflected in the composition of the soil microbial community. A considerable amount of soil and organic substrates passes through the gut of the earthworms in the process of feeding and movement through the soil. The yearly production of coprolites by earthworms in temperate zone soils constitutes an average of 40–50 t/ha [20]. Whenever it passes through the gut, the microbial community is transformed. The scale of the effect of earthworms on microbial communities has not yet been assessed.

In conclusion, we would like to note that the selective effect of the gut fluid of the earthworms on soil microorganisms is important for animal feeding, the maintenance of the homeostasis of the gut microbial community, and the formation of microbial communities in soils.

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