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Particle-dependent reproduction and exogenic biopolymer secretion of protozoa co-cultured rotifers

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ABSTRACT

The rotifer-specific exogenic biopolymer, named Rotimer and its related molecular processes are affected by physical and chemical factors (e.g., temperature, pH or metal ions); however, the study of biological influences (e.g., the presence protozoa) concerning the particle-dependent reproduction (egg laying) and 'biopolymer producing capacity' (BPC) of rotifers is the objective of the present work. Non-planktonic rotifer species (*Philodina acuticornis*, *Adineta vaga*, *Euchlanis dilatata*, and *Lecane bulla*) were studied in paired micrometazoa-protozoa co-cultures involving *Paramecium*, *Diplonema*, and *Amoeba*. These protozoa can be beneficial food sources, enhancing reproduction, or even toxic factors for the above-mentioned animals, but can also function as particle-like mechanical stimulators. Furthermore, current studies reveal that bdelloids, similarly to monogonants, produce filamentous exudate; moreover, the body of bdelloids is covered by their exudate, unlike that of monogonants, especially in the case of *A. vaga*. A mathematical formula was developed as an improved version of a previously published viability marker to characterize the BPC and the relative amount of produced exudate in different conditions.

Rotifer species secreting biopolymers appear to be a general trait indicating a common evolutionary background (e.g., calcium- and particle dependency) of such molecules; therefore, the BPC becomes an experiential sublethal influencing marker to these micrometazoans.

1. Introduction

The cosmopolitan rotifers occur in saline and fresh water, soil, or arctic and high-altitude ice sheets [1]. These exceptional micrometazoans are widely accepted ecological indicators [2] and experimental animal models in numerous fields of exploratory research, such as aging [3], lifespan [4], space exploration [5], neurotoxic aggregates-targeted catabolism [6], toxicology and viability [7,8], evolution and horizontal gene transfer [9] or pharmacology and drug research [4,10]. In addition, they have recently been gaining more relevance related to the *micro*-in vivo OMICS (genomics, transcriptomics, proteomics, glycomics) methodologies [11,12] and environmentally friendly industrial technologies (e.g., source of raw materials). Exploring and investigating variable natural products is one of the main topics of current sciences e. g., biotechnology and medicine. The fact that some monogonant rotifers

are also capable of producing special biopolymers [13] was recently discovered and published for the first time by our team. Due to its novelty, this family of the viscoelastic and proteinous rotifer-specific exudates, named Rotimers, is not yet fully revealed (e.g., physiological background or structure and components).

As natural and degradable organic materials, with a versatile and diverse structure, biopolymers can be used in almost all scientific fields, from nature conservation technologies [14] to industrial nutrient production [15], in the delivery of genes and drugs [16,17], or for the production of nanoparticles in translational biomedicine [18,19]. Among the biopolymers, the Rotimer family is an evolutionarily formed, phylogenetically ancient (presumably more than a few hundred million years old) and protein-like multifunctional molecules, which formation is a uniformly and strictly calcium-dependent process [20]. The preliminary measurements of species-specific Rotimers have shown diverse,

multilevel and promising bioactivities such as the inhibition of the cancer neuroblastoma cell proliferation, anti- and disaggregating effect against neurodegeneration-related beta-amyloid peptides or blocking the algae and yeast cell movement [13,21]. The biopolymer secreting ability has yet been described for monogonant species; therefore, the existence of biopolymers in bdelloids is one of the crucial questions of the present study. Furthermore, we also intended to investigate the form in which bdelloids produce any type of exudate. Three types of Rotimer, namely glue-, film- and fiber-like forms had already been differentiated. The Rotimer producing capacity of rotifers is also a relevant marker of sublethal viability and sensitivity. A limited mathematical description of this natural phenomenon was previously created [13] where the particular formula only compares a spectacular web of conglomerates produced under uniformed and standardized conditions, narrowed down to species and particle-type inducers. It would be more advantageous to use this 'Rotimer-Inductor Conglomerate' (RIC) creation universally, quantitatively, and independently of changing parameters as a novel indicator in the model system of rotifers. In this present work, we also aim to find solutions for these aspirations.

The particle dependence of exudate production process was determined [13,20], where epoxy-metal beads, carmine- and urea crystals, micro-cellulose, and other inducers with a diameter of 2–50 μm were tested. The time of RIC production range from a few minutes up to 2 h, depending on the species used for the process, the type of inductor, the surface area, and the number of individuals. These influencing parameters also reveal that there are no simple methods available to compare biopolymer production between and within species; however, it will be essential for comprehensive experiments in the future. Only indirect experimental data exist on the composition and structure of Rotimer, nonetheless, this objective is one of the most important and interesting questions to answer in the future.

Several studies have already explored the factors influencing the reproduction and egg-laying capacity of rotifers. However, it is important to highlight the effects of other organisms on their populations, both in natural and laboratory conditions, during monitoring and testing self-preservation (nutrition) and species maintenance (egg-laying) of these animals. Such influencing biological factors are, for instance, different protozoa species, which may be present in the most varied forms and amounts in the natural aquatic microenvironments of rotifers. Concerning the ability of ciliates influencing rotifers with their metabolic products, called allelochemicals, has been shown [22]. In addition, other research groups highlighted that there are even more complex chemical regulations among aquatic communities, such as the effect of rotifer-specific surface glycoproteins (e.g., mate recognizing pheromone) or tocopherols, which affect sexual reproduction. [3].

As for representative examples from the world of protozoa in laboratory conditions, cellular organisms from the *Paramecium*, *Diplonema*, and *Amoeba* genus were used. Freshwater rotifers were applied in related co-cultures, since we were only able to detect filamentous exudate secreted by them. To carry out a comprehensive analysis, two bdelloid (*Philodina acuticornis* Murray and *Adineta vaga* Davis) and two monogonant (*Euchlanis dilatata* Ehrenberg and *Lecane bulla* Gosse) non-planktonic species were applied, which allow us to study the effect of protozoal co-culturing in a sufficiently large combination, thus providing a complex picture of their specific interactions. A similar ecological study was performed, where Rotifers were investigated as predators of heterotrophic flagellates and ciliates [23]. At the same time, microscopic organisms were also investigated with co-cultured bacteria, algae, and certain monogonant rotifers, particularly *Brachionus plicatilis* [24]. The specific interactions of this well-known rotifer with Antarctic prokaryotes [25] and *Candida rugopelliculosa* yeast [26] have been widely studied in mixed cultures.

Despite other experiments published in academic literature the current work differs in its concept. The long-term controlled coexistence of rotifer-protozoa cultures and the effect of these combinations on the reproduction, viability and biopolymer production of the micrometazoans have not yet been studied in these contexts. The impact of rotifers or other micrometazoans on the natural environment seems surprisingly wide-ranging [27,28]. The existence and the amount of rotifers in nature and their above-mentioned secreted bioactive exudate may also play a globally decisive ecological regulatory and executive function. Considering the high interaction capability of these animals and their Rotimers with other species and materials, the study of protozoa-influences on the reproduction and biopolymer production of rotifers is the objective of the present work.

2. Material and methods

2.1. Materials

Materials applied in this work were the following: yeast (*Saccharomyces cerevisiae*; EU-standard granulated instant form, cat. no.: 2-01-420674/001-Z12180/HU); algae (*Chlorella vulgaris*; BioMenu, Caleido IT-Outsource Kft.; cat. no.:18255); from Sigma-Aldrich: FBS (cat. no.: F7524); from Merck: powdered Carmine crystals (Natural Red 4; cat. no.: 2233), distilled water (DW; Millipore SAS, Direct-Q 3 UV, ultrapure; type 1; Molsheim, France); from Lonza: DMEM (cat. no.: BE12-604F); from Corning-Costar: 24-well plate (cat. no.: 3524); from Greiner Bio-One GmbH: Petri dish (cat. no.: 430167), flask (cat. no.: 430168); from Life Technologies AS: DynaMag-2 magnet (cat. no.: 12321D), Dynabeads M-270 superparamagnetic epoxy-metal beads (cat. no.: 14301); from Gelman Sciences: Acrodisc 13 filter, Low Protein Binding (pore diameter: 0.2 μm; cat. no.: 4454); universal plastic web (pore diameter: 50 μm); standard medium (mg/L): Ca^{2+} 30; Mg^{2+} 15; Na^+ 3.2; K^+ 0.5; HCO_3^- 150; SO_4^- 2.5; Cl^- 1.4; NO_3^- 4.5; F^- 0.01; SiO_2 8; pH = 7.5; conductibility (20 °C): 395 μS/cm.

2.2. The origin of model organisms

The experiments were performed on bdelloid (*P. acuticornis* and *A. vaga*) and monogonant (*E. dilatata* and *L. bulla*) rotifer species, and on *Paramecium*, *Diplonema*, and *Amoeba* protozoa genus; therefore, no specific ethical permission was needed according to the current international regulations. The species have been maintained and cultured in a standard laboratory environment for several years. These conditions and the origin (Red Cross Lake, Szeged, Southern Great Plain, Hungary; GPS coordinates: 46° 16′ 25″ N; 20° 08′ 39″ E; early summer) of the above-mentioned species were precisely described previously by Datki et al. [13]. Protozoa genus, derived from the original habitat of the monogonants were selected, thus making the common source of co-culture modeling more authentic. The monoclonal population of each species was started from a single individual and then identified by zoological experts. In the case of the protozoa, no specific species were identified, as it would have been inaccurate to do so based only on morphology; however, the genus level was identified in each case.

2.3. Protozoa and rotifer culturing and harvesting

Every culturing and measurement was performed in a constant environment (24 °C, pH = 7.5, 40–45% air humidity, in standard media and 12:12 hour dark-light cycle). The cultivation of rotifers has been continuous for years, while the application time of a given flask takes up to 2–3 weeks, depending on the reproduction rate of the individuals. To provide uniformly standard food of cultures, a mixture of ultrasonicated and homogenized baker's yeast and alga was used after heat-inactivation and filtration (final amount 600 μg/mL; diameter of particles ranged 8–12 μm).

In the case of the free-floating protozoa, i.e., *Paramecium* and *Diplonema*, the individuals were filtered with a plastic web from any debris, concentrated by sedimentation and then pipetted into the relevant wells for the rotifers. In the case of *Amoeba*, their transfer was preceded carefully by mechanically scraping and pipetting to separate them from

the plastic surface temporarily.

Isolation of bdelloid and monogonant rotifers is performed by different methods, due to the fact that bdelloids follow a primarily adherent behavior while the members of the other subclass swim freely. The working volume in the wells (1.8 cm^2 well-area) was 1 mL of the standard medium uniformly. In both cases, it is an adaptation of an earlier methodology [6,8].

Before harvesting bdelloid rotifers their medium was changed, without feeding. The culture flasks were placed at -75 °C for 3 min for rapid cooling (2–4 °C) in order to dissect attached rotifers from the plastic surface on ice. The medium was decanted in petri dish, from which the contracted animals were distributed equally in the wells (10 $\pm$ 1 rotifer/well) that was then left for 30 min (warming of the medium at room temperature) to let healthy animals attach to the bottom. Monogonant specimen were collected and transferred to wells with a pipette, supervising their standard number (10 $\pm$ 1) in the wells under a microscope.

2.4. Experiential monitoring

2.4.1. Photo recordings

The relevant rotifer species (Fig. 1) were photographed (Nikon D5600, DSLR, RAW-NEF, 25 MP, ISO 100; Nikon Corp., Kanagawa, Japan) under inverted light microscope (at 63× and 400× magnification; Leitz Labovert, Wetzlar, Germany). The animals were paralyzed with carboxygenated (5% CO_2) standard medium for 5 min during photo recordings. The processes of all experiential measurements were label-free optical monitoring where rotifer-specific parameters were examined.

2.4.2. Reproduction

The reproduction rate (changes in the number of rotifers) over a 10-day period was examined alone or in the presence of the relevant protozoa species (Fig. 2). The 'number of rotifers alive' (NRA) parameter characterizes the measurements starting with 10 $\pm$ 1 rotifer/well ($n =$ 12 well/species). Number of protozoa entities constantly was 10 $\pm$ 3 in 0.01 mm^2 area (in homogenic dissociation). The rotifers were feed with standard food in the relevant wells. Before feeding, the supernatant of the wells was carefully replaced every two days with a plastic filter-equipped (pore diameter: 50 μm) pipette to avoid the loss of rotifers

in contrast to protozoa. As a consequence, the passively removed amount of cellular entities from wells was replaced by transferring specimens from their monoclonal culture. If the adverse protozoa proliferated beyond the given number, they were also corrected by the methods described above. With this compensation procedure, the standard number of individuals was maintained, even in co-cultures where they were not consumed by rotifers. Regarding reproduction, all rotifers were tested with all protozoa in each and every combination during the ten-day monitoring period. The number of live rotifers in the wells was counted each day. The criteria applied to living individuals were published by [8]. No animals in the passive (permanently contracted) state were found in the bdelloids.

2.4.3. Rotimer secretion

The study of Rotimer secretion and thus, RIC formation, is a modified version of a previously published method [13,20]. In the present application, the duration of Rotimer production is only 10 min; however, the evaluation and comparison of these biological products have become more sensitive due to the new formula, which is suitable for characterizing the degree of RIC formation. The 'biopolymer producing capacity' index is a relative unit, which was calculated by the following formula: $BPC_i = A/B * C/D * E/F * G$; A: average size of conglomerates (μm^2); B: average diameter of inductor particles (μm); C: conglomerate-covered area (%); D: induction time period (min); E: full space area (cm^2); F: starting rotifer number per area; G: longest filament of conglomerate (mm). Some of the parameters were the same (B = 2.8; D = 10; E = 1.77) or in some cases variables (A, C, G) during this project.

Before RIC production, the experimental conditions and the amount of protozoa during the co-culturing period (10 days) was similar to the reproduction-related experiments, e.g., feeding the rotifers in the wells with standard food every two days. Each animal species was assigned to a favorable (+) and an unfavorable (−) protozoa in the following combination: *P. acuticornis* and *E. dilatata* with *Diplonema* (+) or *Amoeba* (−); *A. vaga* and *L. bulla* with *Amoeba* (+) or *Diplonema* (−).

The RIC formation by selected live rotifers was performed identically in all studies ($n =$ 12 well/species; 50 $\pm$ 3 mature entities/well) during 10 min incubation time, including survived animals which originated from disadvantageous co-cultures. No protozoa were present during biopolymer induction and conglomerate formation at the application of epoxy-metal beads inductor (Figs. 3–4). The final (working)
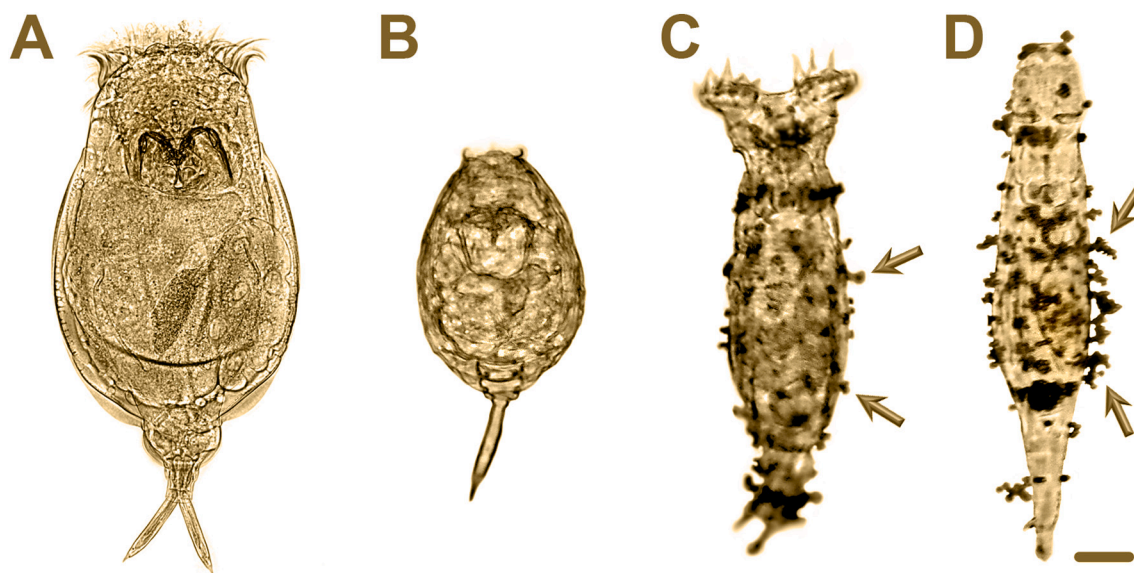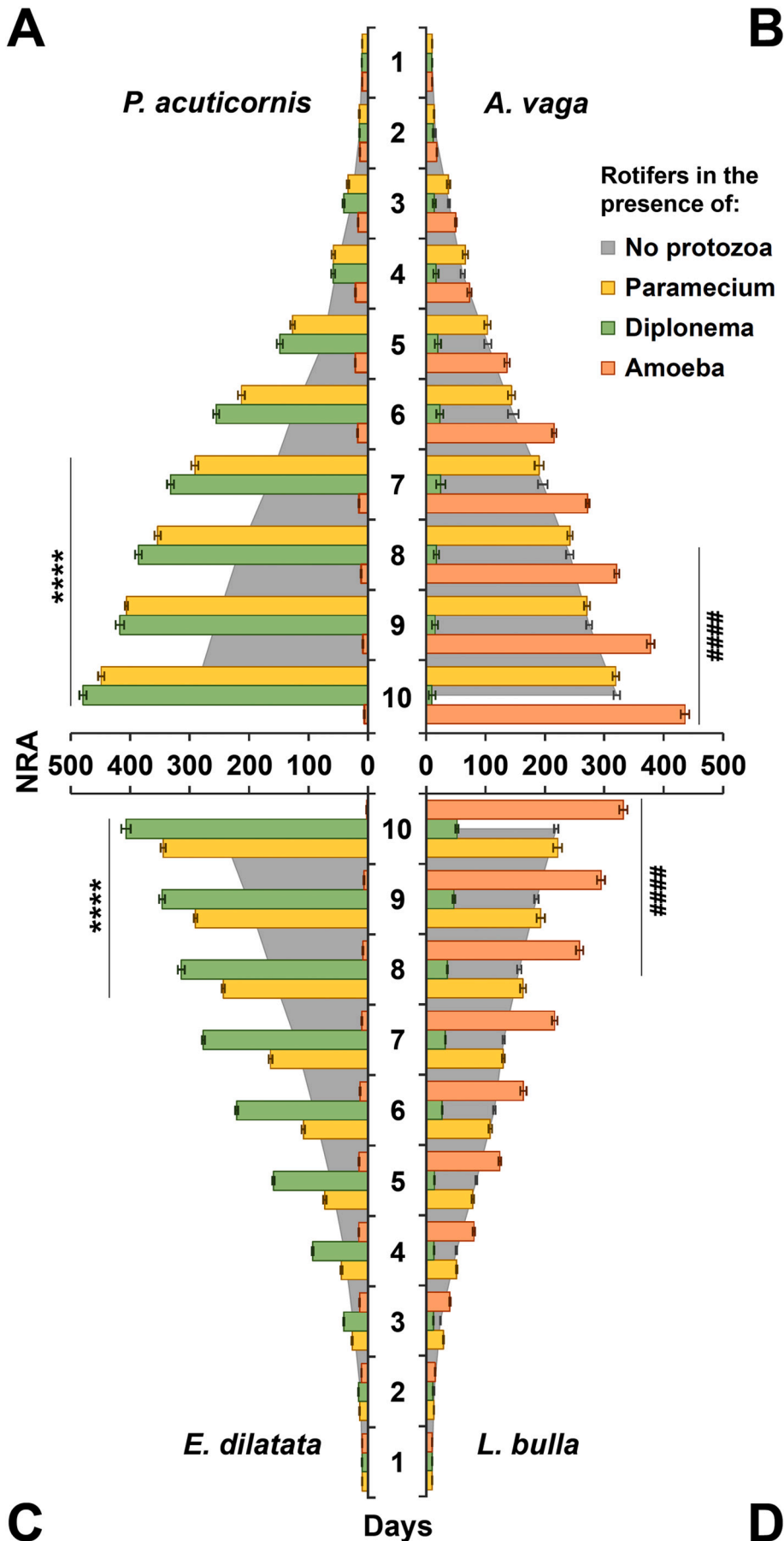

Fig. 1. Presentation of the examined rotifer species. Two monogonant (A: *E. dilatata*; B: *L. bulla*) and two bdelloid (C: *P. acuticornis*; D: *A. vaga*) rotifer species were studied in alternate paired micrometazoa-protozoa co-cultures. Binding and localization of the epoxy-metal beads inductor to the body of bdelloids is presented (see arrows; C, D). The scale bar is 30 μm.

Fig. 2. Protozoa- and time-dependent reproduction kinetics of rotifers in co-cultures. Bdelloid (A: *P. acuticornis*; B: *A. vaga*) and monogonant (C: *E. dilatata*; D: *L. bulla*) rotifer species were presented without or in paired co-cultures with three types of protozoa (*Paramecium*, *Diplonema* and *Amoeba)*. The number of rotifers alive (NRA) for 10 days interval shows the reproduction rate of rotifers. The error bars represent SEM. One-way ANOVA with Bonferroni post hoc test was used for statistical analysis, the levels of significance are p****, #### ≤ 0.0001. (*, significant difference of the all protozoa-containing culture from the protozoa-free control groups, from day 7 or 8; #, significant difference of the *Diplonema* and *Amoeba* containing cultures from the protozoa-free control groups, from day 8).
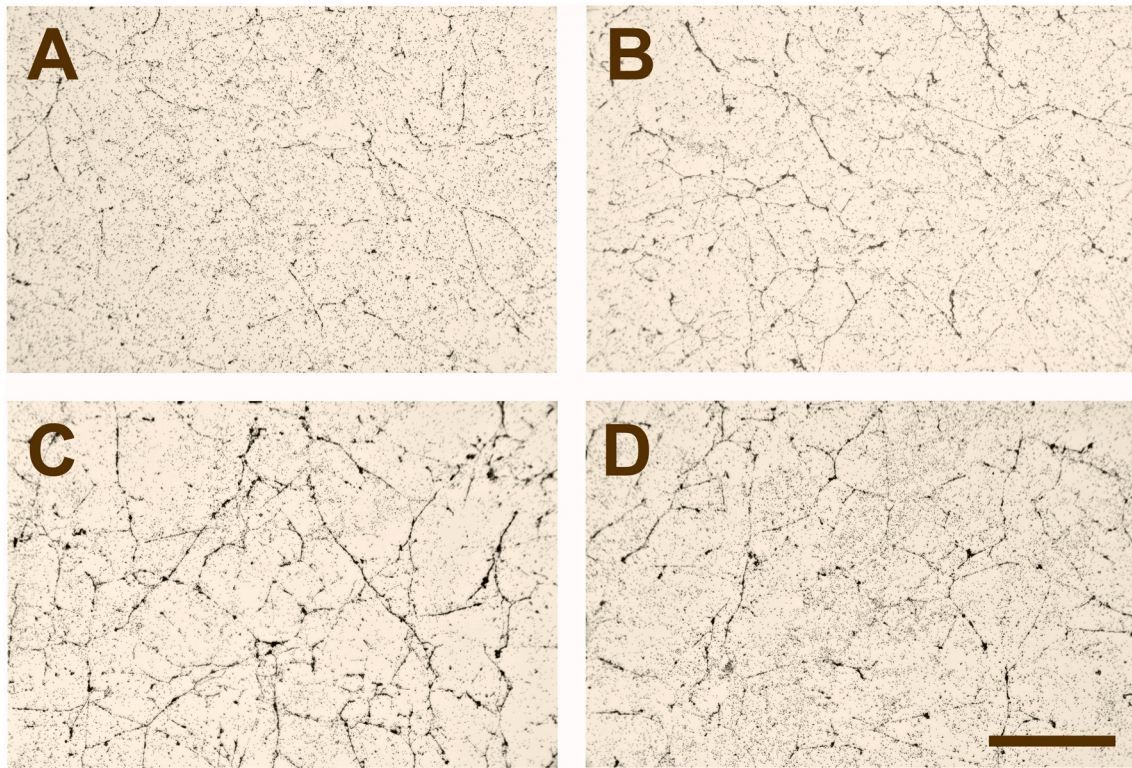
Fig. 3. The rotifer species-specific RIC versions in different co-cultures. The RIC production by bdelloid (A, B) and monogonant (C, D) rotifers were monitored in standard cultures. The scale bar is 1 mm.
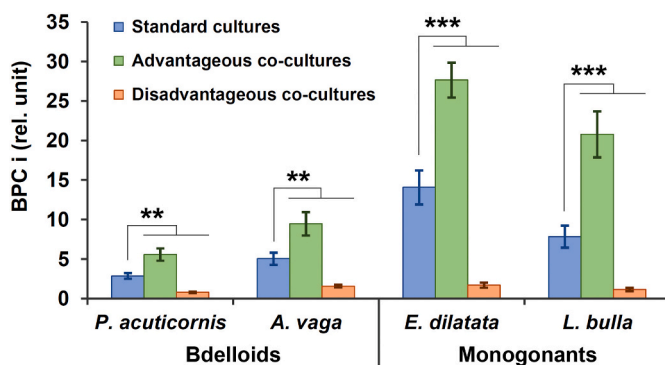

Fig. 4. BPC_i of different rotifer species in advantageous and disadvantageous co-cultures. Bdelloid (*P. acuticornis*, PA; *A. vaga*, AV) and monogonant (*E. dilatata*, ED; *L. bulla*, LB) rotifer species were presented without or in paired co-cultures with advantageous (PA and ED with *Diplonema*; AV and LB with *Amoeba*) or disadvantageous (PA and ED with *Amoeba*; AV and LB with *Diplonema*) protozoa. The index number of biopolymer producing capacity (BPC_i) was applied as comparison formula, including seven different parameters related to the rotifers or their Rotimer (see the text). The error bars represent SEM. One-way ANOVA with Bonferroni post hoc test was used for statistical analysis, the levels of significance are $p** \leq 0.01$; $p*** \leq 0.001$ (*, significant difference of the species-specific co-cultures from the protozoa-free standard control groups).

concentration of this inductor was 50 μg/mL, diluted from 6 mg/mL DW stock solution.

In order to avoid optical interference between adherent animals and RIC monitoring, the plates were placed at 45 °C surface for 5 min. As a result, the rotifers released the surface and so, they could be removed along with the supernatant by careful pipetting. In this adapted and modified method, the desiccated (30 min) RIC patterns (from the center region of the wells), detected by light microscopy, were photographed (Nikon D5600). The digital raw photos were converted in a black and white graphical tiff format (threshold; 1 μm = 2.46 pixel; 8-bit; 6000 × 4000 pixel the full picture). These images were analyzed with ImageJ program (Wayne Rasband, USA), extracting data related to the conglomerate-covered area and the average size of this complex units.

2.4.4. Particle-dependent egg laying

The egg-laying of the rotifers was monitored at 5-day intervals, the old and the generated new eggs were checked and quantified daily with digital photos, then their total number was summarized at the end of this period (Fig. 5). Nonetheless, a two-day starvation period preceded the monitoring in order to get egg-free animals ($n = 12$ well/species in 24 well-plate; 25 ± 2 rotifers/well in the initial time).

No protozoa were present when measuring the number of eggs laid. Therefore, they were performed under standard conditions consistent with non-protozoa-containing controls. The proportion of viable eggs from which rotifers hatched was around 90%, corresponding to a healthy culture (data not shown).

The micrometazoans were feed with relevant food (standard: 600 μg/mL; artificial: diluted) two times during 5 days (at the beginning and then on day 3) in wells. The artificial food, applied on these animals, was based on the commonly used cell culturing media: DMEM (dilution: 70×, from 15 g/L to 212 mg/L) substituted with FBS (dilution: 120×).

Various experimental groups were set up to induce and study Rotimer production: 1. standard food as inductor (yeast and algae cell skeleton together with their cytoplasmic content); 2. inductor-free natural extract (yeast and algae cell content only, cell skeletons filtered out the by Acrodisc 13 filter); 3. natural extract (cell content only) with different inductors such as carmine crystals, epoxy-metal beads and powdered micro-grains of sand (maximum diameter 50 μm); 4. inductor-free artificial medium (DMEM with FBS); 5. artificial medium with different inductors such as carmine crystals, epoxy-metal beads and micro-grains of sand. Rotifers do not consume these particles as food.
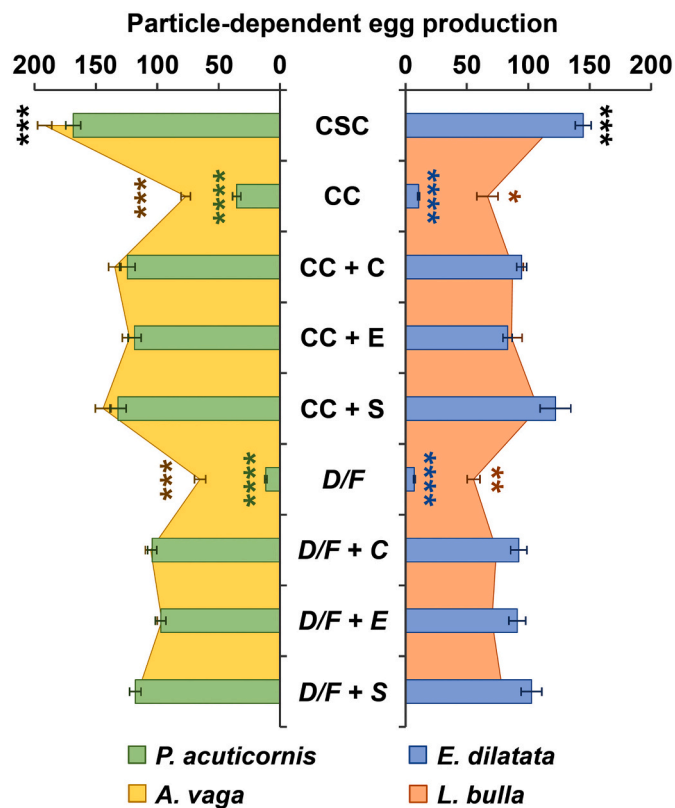
Fig. 5. Various inductors stimulated egg production of different rotifer species (*P. acuticornis*, *A. vaga*, *E. dilatata* and *L. bulla*). The investigated experimental groups were the following: cell skeleton and content (CSC), cell content only (CC), cell content + carmine (CC + C), cell content + epoxy (CC + E), cell content + sand (CC + S), DMEM/FBS (D/F), DMEM/FBS + carmine (D/F + C), DMEM/FBS + epoxy (D/F + E), DMEM/FBS + sand (D/F + S). The error bars represent SEM. One-way ANOVA with Bonferroni post hoc test was used for statistical analysis, the levels of significance are p* ≤ 0.05; p** ≤ 0.01; p*** ≤ 0.001 and p**** ≤ 0.0001. (*, significant difference from all groups of the same species in which an inductor is present).

2.5. Statistics

Statistical analysis was performed with GraphPad Prism 7.0b software (GraphPad Software Inc., La Jolla, CA) using one-way ANOVA with Bonferroni post hoc test. The error bars represent the standard error of the mean (SEM). Using the above-mentioned statistical test, 99% confidence level (1-alpha = 0.01) was applied and 1% error was handled in this analysis. The homogeneity of variances was checked, data in these studies were parametric, and they were found suitable for one-way ANOVA. The different levels of significance are indicated as follows: p* ≤ 0.05; p** ≤ 0.01; p*** ≤ 0.001 and p****, #### ≤ 0.0001. All statistical relations and comparisons between the presented groups are defined in the given figure legend.

3. Results and discussion

Microscopic invertebrates, such as rotifers, plathelminthes or nematodes, are emerging ecological, physiological, and molecular models in current biological sciences [29,30]. Their adaptive phenotypic plasticity makes them suitable for both holistic and targeted studies [31]. In our previous works [20,32], similarly to the current project, four rotifer species (optimized for routine laboratory culturing), two bdelloid (*P. acuticornis* and *A. vaga*) and two monogonant (*E. dilatata* and *L. bulla*) types were systematically used. These species are adequate representatives of the freshwater rotifers worldwide. Their co-culturing with protozoa may serve as a model to better understand the interaction and

relationship between the species in their natural environment. As an example for interspecies facilitation, it is particularly advantageous for rotifers that unicellular animals consume the various prokaryotes, thereby providing food and environmental regulators (e.g., hormones or pH) by themselves and their metabolites. [33,34]. In the present work, the effect of protozoa (interpreted as living and mechanical factors) on the reproduction and biopolymer production of rotifers was investigated. The genus (*Paramecium*, *Diplonema*, and *Amoeba*) of protozoa were selected more widely in order to cover the group of these cellular organisms. Species-specific identification was not required for the protozoa, since they have typical morphology and behavior, which were accurately determined. There are substrate-attached and free-moving types.

As for the rotifers investigated in this study, monogonants (Fig. 1A, B) perform a swimming motion, while bdelloids (Fig. 1C, D) tend to live stationary. Other characteristics, such as Rotimer secreting property was first attributed [13] to monogonants, but subsequent and current results have shown that bdelloids also produce this natural bioproduct. Epoxy-metal beads, used for inducing secretion, showed that the body of bdelloids, in contrast with monogonants, is also covered by some exudate. The beads were highly bound to the bodies of *P. acuticornis* (Fig. 1C) and even more to the *A. vaga* (Fig. 1D).

Protozoa had a completely variable effect on the different rotifer species and their reproduction (Fig. 2) in co-culture conditions. Compared to the protozoa-free control groups, *Paramecium* and *Diplonema* were preferred for *P. acuticornis* (Fig. 2A) and *E. dilatata* (Fig. 2C). At the same time, the presence of *Amoeba* proved to be particularly toxic for these animals. It is noteworthy that in the case of *A. vaga* (Fig. 2B) and *L. bulla* (Fig. 2D), *Amoeba* had a stimulating effect, while *Diplonema* drastically reduced their reproduction.

Rotifers consume various microorganisms in the different co-cultures, such as protozoa [23,35]. In line with the above, the number of protozoa in a given culturing well was halved in one day (data not shown) during our experiments, and this status was compensated daily by adding extra protozoa to each well. The number of adverse protozoa was also kept technically constant in the respective wells, although the relevant rotifers did not consume them.

The ambivalent and diffuse effects of protozoa on the reproduction of rotifers may be explained by the different dietary habits of these animals. Rotifers that primarily filter food from the medium (*P. acuticornis* and *E. dilatata*) prefer floating protozoa (*Paramecium* and *Diplonema*), while those collecting from the bottom (*A. vaga* and *L. bulla*) prefer *Amoeba*. It is especially noteworthy that the significant inhibition of the growth of rotifers and the decrease in the number of individuals in the above-mentioned negative co-cultures also indicate a chemical-biochemical interaction with the protozoa or the substances produced by them.

Rotifers can also actively contribute to the ecological balance of the aquatic environment through the production of their biopolymers, which are regulated by physical parameters and chemical agents. Furthermore, during the investigation of biological factors, in addition to reproduction, the Rotimer-producing ability of the currently used micrometazoa species was examined as well in co-cultures. It was found that bdelloids (Fig. 3A, B), like monogonants (Fig. 3C, D), form exogenic filamentous biopolymer. Revealing the Rotimer secretion of these subclasses came as a surprise raising the question, why this phenomenon and ability has not been noticed so far? Measurements show that this group of rotifers is not equally sensitive to different particles used as mechanical inducers; furthermore, less displacement over time results in less exudate secretion. Nevertheless, the most determining factor in the problematic detection of filamentous biomaterial is that in most cases, the RIC web is only visible approximately in the first 15 min during the induction period. The created conglomerate structure is completely disrupted by the movement of bdelloids in a relatively short time. This was also the reason for the current methodological implementation: we examined and compared the status of RIC in different species only 10

min after induction, in contrast to our previous studies [20] where the time interval was manifold. The formation of the conglomerate depends on several environmental factors; however, it fundamentally changes and saturates exponentially. Current processes show similar time-dependent kinetics in measurements.

As for bdelloids, the quality and quantity of conglomerate produced in a time unite are lower than that of the species used in the case of monogonants. RIC formation was increased by rotifers being co-cultured with beneficial protozoa (*P. acuticornis* and *E. dilatata*, with *Diplonema*; *A. vaga* and *L. bulla*, with *Amoeba*), while it was significantly reduced in an unfavorable association (*P. acuticornis* and *E. dilatata*, with *Amoeba*; *A. vaga* and *L. bulla*, with *Diplonema*). It is exciting to note that the subclasses (bdelloids or monogonants) are not specific to particular types of protozoa. Although the background and specificity of the positive effects can mainly be explained by the diet of the rotifers, it is assumed that there is a biochemical competition between micrometazoans and protozoa in the case of species-specific negative influences. The molecular mechanism of these phenomena needs further investigation, which is beyond the scope of the present work. For the quantitative evaluation of representative recordings, a previously published [13] mathematical formula was further developed. Currently, the biopolymer secretion ability of rotifers (Fig. 4) has been characterized by considering seven different parameters (average size of conglomerates, average diameter of inductor particles, conglomerate-covered area, induction period, whole space area, starting rotifer number per area, and longest filament of conglomerates). The advantage of this updated and modified BPC-formula by its index value (BPC_i) is that the rate of the RIC production is comparable in any conditions (e.g., different inducers and number of animals or relevant area). This simple experiential optical imaging marker can serve as a label-free detection method, which could be one of the defining tools of Rotimer research in the future. BPC_i calculation also provides a possibility to use it as a rapid and sensitive sublethal influencing and viability marker of rotifers.

The tested categories and co-culture conditions were applied, and quantitative analysis of the results was calculated. As for bdelloids, there was no change between regular and co-cultured groups in terms of quality parameters (average size of conglomerates and longest RIC fiber) in contrast to quantitative characteristics (conglomerate-covered area). At the same time, in the case of monogonants, there was a significant difference in all three measured parameters. In the mathematical analysis of the RIC recordings (Fig. 4), the results and correlations described earlier on were found. Significant increase or decrease in BPC_i was detected in the co-cultures, depending on the presence of favorable or unfavorable protozoa.

In nature, rotifers can generally be affected by other types of rotifer species as well as invertebrates from different categories (e.g., Turbellaria or Copepoda), which either function as dietary competitors or as predates [36,37]. The exploratory level of these interspecies interactions is not investigated in the present work related to reproduction and BPC, nevertheless, exploring the possible role of Rotimer exudates in other species may be an exciting topic for future studies.

Based on the obtained results, it can be concluded that protozoa living in nature, in addition to serving as food, can also function as special regulators for the viability and activity of both rotifer subclasses. The presence of various debris, inanimate cell skeletons, or even the body of cellular organisms can act as physical stimulators, which the rotifers sense with their highly sensitive cilia. If these tested formulations and protozoa organisms are uniformly considered 'mechanical granules', we can conclude that the egg-laying of rotifers is most probably particle dependent, and the processes are linked with biopolymer production. In the absence of irritating particles, egg production was low, despite receiving nutrient-rich natural (filtered standard food suspension) or artificial (diluted cell culture media) foods (Fig. 5). When these organic solutions were added to the animals, supplemented with a physical particle (carmine crystals, epoxy beads, or sent), they began to lay eggs intensively, regardless of the type of the tested species. The

phenomenon of particle-dependent egg production is a novelty in rotifer research. Rotifers (*A. vaga* and *L. bulla*) that pick up much of their food from the underlaid are less particle-dependent in terms of ovulation. In contrast, those that feed from the fluid space (*P. acuticornis* and *E. dilatata*) require living or inanimate substances that irritate their cilia. There is no difference in the background of this phenomenon according to the given species belonging to the bdelloid or monogonant subclasses; at the same time, both types have more sensitive or less sensitive conditions. The fact that protozoa, which are thoroughly stimulating to a particular type of rotifer are fatally harmful and even toxic to another species can be considered a novelty. In a comprehensive evaluation of these results, similar trends and a high degree of correlation between rotifer reproduction (Fig. 2) and BPC_i (Fig. 4) were found.

Several studies describe [27,38] the phenomenon that rotifers use their cilia to direct substances (e.g., debris), needed for survival, growth, and reproduction into their digestive system. In contrast, no one has yet published the fact that in the case of particle-free dissolved food, the rate of ovulation is significantly reduced. This situation is even valid in the condition when the suspension provides adequate nutrition and a diverse source of organic matter for the animals. Academic literature had described the egg-laying of *E. dilatata* as highly dependent on environmental factors, such as the behavior of one's own and other species, and the site and environment of hatching [39]. It also appears that not only the facts examined in this work influence ovulation, but the research target outlined has also proved to be essential.

According to the current results it can be assumed that the rate of egg formation is reduced in the absence of particle-based stimulation; however, mechanical irritation is required for rotifers to produce biopolymer, which exudate serves as a fixative to their eggs. This secreted biomaterial by *E. dilatata* is well observed in cultures where their eggs are attached to inserted yarns or even to each other (Fig. 6). Therefore, it can be stated that the particle-dependent biopolymer secretion and egg-laying is a strongly related phenomenon on this *micro*-in vivo level. However, we do not yet know whether exogenic Rotimers, as substances in contact with eggshells, also have a special regulatory effect in ontogenesis of rotifers.

4. Conclusion

The reproduction and physiological functions (such as BPC) of rotifers are directly influenced by biological environmental factors both in natural and laboratory conditions. For example, protozoa fill various functions, they serve as food sources, toxic agents, competitors, or just mechanically stimulating particles. Rotifers selectively hunt for their favorable protozoa in different species-specific combinations; however, some co-culturing interactions may have negative effects on the tested animals. The BPC of these micrometazoans obviously depends on the type of protozoa present in the relevant culture.

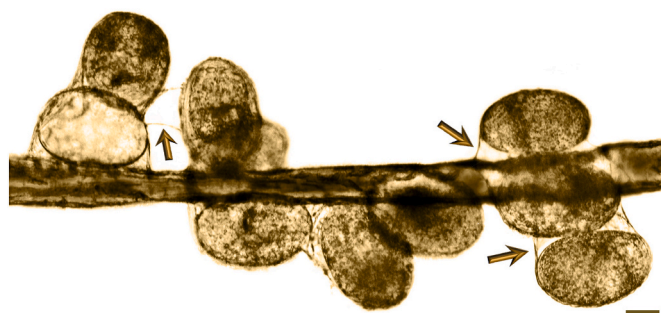The Rotimer secretion is a sensitive sublethal influencing and

Fig. 6. Rotimer fixed eggs of *E. dilatata* rotifers. Representative illustration of the eggs laid by rotifers on the yarn placed in the culture, fixed with their exudate (see arrows). The scale bar is 30 µm.

viability marker of rotifers, described and characterized by an updated and uniformly applicable mathematical formula. Bdelloids, such like monogonants, can also create the filamentous exogenic biopolymer, which fact in an outstanding novelty. The secretion of these exudates is a possible general ability of the Rotifera phylum. The particle dependency of egg production, or in other words the exudate secretion-based egg-laying and fixation performed by rotifers, depends on ciliate-stimulating exogenic particles, especially in the case of monogonants.

The current work is an essential part of the comprehensive studies published on the biopolymer of rotifers. We were the first to report about the existence of these natural substances and their bioactivity in vivo and in vitro. The multifunctional effects of Rotimers were demonstrated from ecological aspects, through the inactivation of neurotoxic aggregates to the immobilization of cancer cells.

CRediT authorship contribution statement

Zsolt Datki: Investigation, Formal analysis, Writing – original draft, Visualization, Funding acquisition, Project administration. **Rita Sinka:** Resources, Writing – review & editing. **Bence Galik:** Software, Data curation, Funding acquisition. **Zita Galik-Olah:** Validation, Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Ethical approval

All procedures were conducted in accordance with the globally accepted norms: Animals (Scientific Procedures) Act, 1986, EU Directive 2010/63/EU for animal experiments, and the National Institutes of Health guide for the care and use of Laboratory Animals (NIH Publications No. 8023, revised 1978). Furthermore, our animal studies comply with the ARRIVE guidelines. Samples and data were collected according to University of Szeged (Szeged, Hungary) protocols under the supervision of the Hungarian Academy of Sciences. All applicable international, national, and institutional guidelines for the care and use of animals were followed. Our experiments were performed on rotifers; therefore, no specific ethical permission was needed according to the current ethical regulations.

Availability of data and material

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2022.05.020.

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