Biofilm formation initiating rotifer-specific biopolymer and its predicted components

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A B S T R A C T

The rotifer-specific biopolymer, namely Rotimer, is a recently discovered group of the biomolecule family. Rotimer has an active role in the biofilm formation initiated by rotifers (e.g., *Euchlanis dilatata* or *Adineta vaga*) or in the female-male sexual interaction of monogononts. To understand the Ca^{2+}- and polarity-dependent formation of this multifunctional viscoelastic material, it is essential to explore its molecular composition. The investigation of the rotifer-enhanced biofilm and Rotimer-inductor conglomerate (RIC) formation yielded several protein candidates to predict the Rotimer-specific main components. The exudate of *E. dilatata* males was primarily applied from different biopolimer-containing samples (biofilm or RIC). The advantage of males over females lies in their degenerated digestive system and simple anatomy. Thus, their exudate is less contaminated with food and endosymbiont elements. The sequenced and annotated genome and transcriptome of this species opened the way for identifying Rotimer proteins by mass spectrometry. The predicted rotifer-biopolymer forming components are SCO-spondins and 14–3-3 protein. The characteristics of Rotimer are similar to Reissner's fiber, which is found in the central nervous system of vertebrates and is mainly formed from SCO-spondins. This molecular information serves as a starting point for its interdisciplinary investigation and application in biotechnology, biomedicine, or neurodegeneration-related drug development.

1. Introduction

Micro-metazoans (animals smaller than 2 mm) play a central role both ecologically and molecularly in the dynamic processes of the natural habitat [1]. Their existence and extensive inter- and intra-species interactions (such as symbiosis or predator-prey relationships), along with their adaptive phenotypic plasticity in response to environmental conditions, are essential for the functioning of the entire biosphere [2,3]. As a result of adaptation and survival, most of these animals can produce specialized exudates (e.g., mucins), often releasing them into their environment [4]. Among these substances are well-known and widely applied (even artificially) homogeneous and heterogeneous biopolymers (e.g., spider silk, cellulose, chitosan, casein, collagen, gel-forming mucins, or von Willebrand factor polymers), as well as complexly structured and organized biofilms formed collaboratively by different prokaryotic and eukaryotic species [5–8]. One of the fundamental requirements for the occasional beneficial or harmful biofilm formation is the secretion of various natural substances into the environment, named

extracellular polymeric substances [9,10], and their interaction with various molecules or environmental agents, e.g., with metal ions [11,12]. The role and impact of these complex molecular organizations are inexhaustible. They can yield different approaches in interdisciplinary research [13,14], both in terms of defense against biopolymers (e.g., inducing oxygen depletion in natural waters) and their potentially valuable applications (e.g., degradation of organic materials or flocculation-based water purification) [15].

Various representatives of the living world, including prokaryotes, protozoa, and metazoans, can produce different naturally biodegradable polymers [16]. Biomolecule polymerization can occur intra- (e.g., microtubules) and extracellularly (e.g., cellulose), as well as at the inter- and external boundaries of a living organism (e.g., spiders) [17]. Rotifers can also be classified as micrometazoans capable of secreting exogenous substances by their rotifer-specific biopolymer, namely Rotimer. They can produce a filamentous or glue-like exudate into the external environment through induction and stimulation by physical particles, creating a network-like Rotimer-Induction Conglomerate (RIC) complex. During live microscopic monitoring, it was observed that the animals formed RIC around their mouth [18]. This ability is present in monogononts (e.g., *Euchlanis dilatata* and *Lecane bulla*) and bdelloids (e.g., *Philodina acuticornis* and *Adineta vaga*) as well. The Rotimer is a vital proteinaceous viscoelastic material with relatively high tensile strength, and its acute and rapid external formation is strictly calcium ion (Ca^{2+})-dependent. Furthermore, precipitation during the process is indicated by the fact that when EDTA is present during the RIC formation (equivalent to Ca^{2+} content), the value of the biopolymer-producing capacity index (BPC_i) decreases significantly to a barely measurable value; however, when the chelator is added to the already existing RIC web, it does not break down this biopolymer structure [18]. The essential Ca^{2+} becomes incorporated into RIC, presumably stabilizing the polymerization of the Rotimer. However, in a stricter sense, this metal ion is only needed for precipitation, and the partial exudates that adhere to each other in colloidal dimensions are stable even without the Ca^{2+}. The RIC production is influenced and sometimes regulated by environmental physicochemical factors (e.g., temperature, osmolarity, pH) and biological factors (e.g., presence of protozoa) [19,20]. Protozoa, such as amoebas, diplonemas, and ciliates, can have different effects on rotifers, and these interactions can be species-specific, either beneficial or detrimental. Rotimer, due to its highly adhesive properties, possesses natural multifunctional bioactivity, including inhibition of adherent and suspended cell motility, egg adhesion, trapping of food, and water purification through flocculation. Despite numerous facts, the role of Rotimer in the life of rotifers and the ecological environment still needs to be fully explored.

Rotifers, as experimental (e.g., aging, lifespan, toxicology, viability, or phenotypic plasticity) models [21–23], are applicable in classical supramolecular studies, modern multi-omics (e.g., genomics, proteomics, or metabolomics) and horizontal gene transfer research, DNA-repair, or interdisciplinary investigations (neurodegeneration, drug development) [24–26]. Recently, a novel concept has emerged as one of the research directions specific to rotifers, suggesting that these animals possess a unique ability to inactivate and catabolize human-type neurodegeneration-related aggregates (beta-amyloids, alpha-synuclein, and prions) [27]. In their natural environment, numerous neutral aggregates and conglomerates can be found as remnants of decomposable organic waste. The utilization of aggregated toxins that accumulate in the nervous tissue of vertebrates [28] as regular food sources by rotifers was surprising. This property suggests an evolutionarily developed capacity for metabolizing adequate, yet complex and stable biomaterials [29]. Unfortunately, the molecular mechanisms underlying this ability are still unknown. From human-related studies, it is well-known that the biochemical degradation of beta-amyloids can only occur through enzymes (e.g., insulin-degrading enzyme, matrix metalloproteases, or Neprilysin) if the aggregated conformation (prior to protease activity) is influenced by structure-modifying molecules (e.g., beta-sheet breakers,

hormones, anticoagulants) [30–33]. An ideal and efficient strategy against aggregates or different conglomerates requires complex solutions with a dual mechanism of action that destabilizes and degrades the molecular targets. In the digestive-based catabolic ability of rotifers, it is plausible to assume that a special biomaterial must play a key role in these processes, loosening or even disassembling the mentioned aggregates and making them accessible to classical degradative enzymes.

Rotifers, as microscopic animals consisting of approximately 1000 cells, are evolutionarily conserved organisms [34]. Besides nutrition and reproduction, one of their most interesting activities is producing exogenous biopolymers. In our previous work [20], we hypothesized that this exudate is utilized as a multifunctional tool, possibly for the metabolism of degradable materials present in their environment. The discovery and study of the Rotimer molecule family led to the investigation of its interaction with human-type beta-amyloid. These measurements revealed that this biopolymer plays a central role in neutralizing and catabolizing the mentioned neurotoxin, thus enabling its utilization as a non-toxic food source [35]. In the in vivo studies, Rotimer-depleted animals induced through multiple particle induction were found to be sensitive to aggregated beta-amyloid treatment, indicating significant toxicity, unlike their counterparts that possessed the biopolymer endogenously (non-depleted). Furthermore, it has been demonstrated that the neurotoxin not only serves as a food source for the rotifers but also acts as a positive factor inhibiting their autocatabolism, meaning that certain released amino acid composition of the aggregate regulates the cellular metabolic processes. In in vitro molecular interaction studies with Rotifer-free molecules, it became evident that the exudate containing Rotimer possesses anti- and disaggregating effects on beta-amyloid [36]. Based on the above, it can be hypothesized that the biopolymer of these animals can induce such a level of conformational change in otherwise stable aggregates that makes them accessible to degradative enzymes.

Although the recently explored 'rotifer-Rotimer-aggregates' correlations have yielded logical, interdisciplinary, and forward-looking results regarding phenomena and effects [37] the molecular mechanism of action of the biopolymer remains unknown. Up until the composition and predicted structure of Rotimer are revealed, it is impossible to interpret and apply these molecules in further targeted studies, such as phylogenetic analysis or drug development in neurodegeneration. Some currently known biopolymers [5,38] have well- characterized structures (such as various spider silks or collagen). However, in some cases, the biochemical composition still remains unexplored, as in the case of Gorgonin (a polyphenol-containing protein-like material). Pallas (1766) was the first to describe [39] this bromine and iodine-containing substance, but its exact composition has remained unknown [16,40]. The current effort to explore molecular composition is not straightforward in the case of rotifers, because during the formation of Rotimer (due to its adhesive properties) it can interact with numerous contaminating molecules (e.g., food, dead individuals, endosymbiotic bacteria), even in a semi-sterile laboratory environment. Their partial degenerated polyploid genome [41,42] is also a complicating factor for their study. The technical basis of this work is the exploration, extraction, and analysis of Rotimer-containing biological samples produced by rotifers, as well as the investigation of additional roles or natural occurrences of the rotifer-specific biopolymer and the prediction of its molecular components. This recently described family of biomaterials may open up diverse research directions in the future, including their particular ecological or industrial function and the evolutionary background information inherent in their molecular structure.

2. Material and methods

2.1. Materials

Materials applied in this project were the following: *Chlorella vulgaris* (cat. no.:18255; BioMenu, Caleido IT-Outsource Kft.) and *Saccharomyces*

cerevisiae (cat. no.: 2–01-420,674/001-Z12180/HU, EU-standard granulated instant form); from Sigma-Aldrich: Micro particles based on polystyrene (size 5 μm; cat. no.: 79633), Laemmli buffer (with double concentration; cat. no.: S3401-10VL), distilled water (DW; Millipore SAS, Direct-Q 3 UV, ultrapure; type 1; Molsheim, France); from Merck: powdered Carmine crystals (Natural Red 4; cat. no.: 2233), sodium azide (NaN$_3$; cat. no.: 822335), anti-tubulin antibody (detyrosinated; cat. no.: AB3201); from Biorbit: anti-SSPO antibody (cat. no.: orb507583); from Proteintech: anti-14–3-3 antibody (cat. no.: 14503–1-AP); from Greiner Bio-One GmbH: Petri dish, 90 × 20 mm (cat. no.: 430167; Frickenhausen, Germany); from CytoOne: Petri dish, 150 × 20 mm (cat. no.: CC7682–3614; StarLab International GmbH, Hamburg, Germany); from Corning-Costar: 24-well plate (cat. no.: 3524); from Life Technologies AS: Dynabeads M-270 super magnetic epoxy-metal beads (cat. no.: 14301), DynaMag-2 magnet (cat. no.: 12321D); universal plastic (nylon) web (pore diameter: 10 and 50 μm); standard culture medium (mg/L): Ca^{2+} 37; Mg^{2+} 17; Na$^+$ 2; K$^+$ 1; HCO$_3^-$ 180; SO$_4^-$ 4; Cl$^-$ 1; NO$_3^-$ 2; SiO$_2$ 6. The difference was compensated in favor of Na$^+$ and HCO$_3^-$ in the Ca^{2+}-free experimental medium. All types of media have a material content of 250 mg/L, pH = 7, and conductivity (20 °C) between 384 and 392 μS/cm. Materials used during rotifer genome and transcriptome sequencing (genomics) or in mass spectrometry (MS; proteomics) are detailed in the respective method descriptions.

2.2. The origin and culturing of model organisms

In the present study, we exclusively used laboratory-cultured rotifers. The experiential and omics measurements were performed on monogonont (*E. dilatata* and *L. bulla*) and bdelloid (*P. acuticornis* and *A. vaga*) species. No specific ethical permission was needed according to the current international regulations. The animals have been cultured in a standard laboratory environment for over eight years [18]. A single mature and healthy individual initiated the monoclonal population of each rotifer species. The origin of the species, as mentioned earlier, was from Red Cross Lake, Southern Great Plain, Szeged, Hungary (GPS coordinates: 46° 16′ 25″ N; 20° 08′ 39″ E).

The permanent culturing was performed in a constant environment (23 °C, pH = 7 in standard culture media; standardly 45–50 % air humidity, and 12:12 h dark-light cycle). The cultivation of rotifers has been manifested in surface-treated (hydrophilic) flasks, while the application period of a given flask takes up to 2 weeks. For the standard food preparation of cultures, a mixture of homogenized and ultrasonicated alga and yeast was used after heat-inactivation (65 °C/4 h) and filtration (particle diameter ranged from 10 ± 2 μm; the final dose was 0.6 mg/mL).

Genome and transcriptome sequencing was performed on *E. dilatata* populations that were rested for 3 days (without food). This interval during the experiment was not stressful for the animals, since it was no different from their usual laboratory living conditions. The entities were isolated and washed using plastic webs with different pore diameters (suitable for the given purpose); then, they were frozen in a water-free but non-dried form (−75 °C) until DNA and RNA preparation. Genomic DNA was isolated using Quick-DNA MiniPrep Plus Kit (Zymo Research), and the total RNA was purified by applying Quick-RNA MiniPrep Kit (Zymo Research).

2.3. Photo and video recordings

Imaging analysis was used to perform microscopic observations and measurements (e.g., swimming speed or exogenic conglomerate web pattern and amount). The investigated rotifer species were photographed and filmed using a digital DSLR camera (Nikon D5600, RAW-NEF, 25 MP, ISO 100; 60 FPS, Full HD resolution; Nikon Corp., Kanagawa, Japan) with an inverted light microscope (at 63× and 400× magnification; Leitz Labovert, Germany). The animals were immobilized using a carboxygenated (5 % CO$_2$) medium (optimized for gas

surplus) during the photo recordings for 5 min. The photos were converted to a black-and-white graphical format (8-bit threshold; 1 μm = 2.46 pixels; complete picture: 6000 × 4000 pixels) to analyze the relevant RIC patterns created by *E. dilatata* rotifers. These digital recordings were analyzed using the ImageJ program (Wayne Rasband, USA) to extract data related to the average size of conglomerates and the area of covered surface by this complex. The experimental measurements involved optical monitoring (label-free) in examining specific parameters related to the individual rotifers.

2.4. Monitoring the E. dilatata female-male characteristics

To specify the components of Rotimer, the *E. dilatata* species proved to be the most suitable samples, especially the males; however, both female and male individuals were applied to determine their species-specific biopolymer composition. The occurrence of male individuals was enhanced by acute cold-stress (Suppl. Fig. 1). Mature females were isolated from the standard culture using a plastic web (pore diameter: 10 μm) and transferred to Petri dishes (90 mm Ø/50 mL standard medium) at a density of 30 ± 5 entities/mm^2. After half an hour of rest, acute cold-stress was applied (15 min/5 °C), followed by one hour of regenerative rest at room temperature. Animals were fed with reduced food quantity (0.2 mg/mL) once and left to rest for 24 h. The eggs laid by the rotifer population adhered to the bottom of the Petri dish, particularly at the edges. The active animals were carefully washed off the next day, leaving only the eggs in the culture. In the case of cold-stressed animals, the number of eggs and the ratio of males increased compared to the untreated control (n = 12/case, Petri dish).

The rotifer swimming velocity (μm/s) was measured by analyzing relevant digital Full HD video recordings. The animals were observed in an optical microscope within a 5 mm diameter field of view, occasionally moving the Petri dish as the movement of the males was relatively fast (1.2 mm/s; n = 12/case, entities). All males were monitored between 2 and 4 h after hatching. Based on our experience, males live for an average of 48 h under the current laboratory conditions, thus making their early-stage examination ideal.

Direct interaction between females and males was examined using selected individuals from both genders. Animals were individually transferred using a pipette to a 24-well plate (50 ± 3 females together with 20 ± 1 male per well), and the interaction period lasted for one hour under reduced lighting conditions. The measured parameter was the number of stable connections (lasting at least 2 min continuously) between genders (n = 12/case, well) during one hour in each respective well.

2.5. Biofilm and RIC induction, detection, and preparation

Determining the composition of Rotimer requires analyzing all samples containing it; therefore, the production and isolation of biofilm and RIC are of primary importance for testing. For biofilm formation, the monogonont species were isolated using a plastic web, while bdelloids were collected using a water splash as a mechanical method. Both external biomaterials (biofilm and RIC) were cultivated in large Petri dishes (150 mm Ø; water column height was 1 cm, with 100 mL) with a relatively high population density (10 ± 1 entities/mm^2). Biofilm formation lasted for five days under varying environmental humidity conditions (ranging from 30 to 70 in 10 percentage point increments), and the amount of food provided was 0.6 mg/mL. A formula was introduced to quantify the amount of biofilm formed on the surface of the culture medium for comparability: 'Biofilm Formation Rate' (BFR; relative unit) = dry mass (μg/cm^2)/top rotifers (%). Dry mass samples were obtained from a prepared (1000 *g*/50 min centrifugation) cell- and particle-free biofilm homogenate (milky white) solution. The supernatant was centrifuged again (35,000 *g*/15 min), and the pellet was dried (at 23 °C and 50 % air humidity) and weighed using a precision balance (BEL Engineering). This quantity was compared and proportioned to the

surface area from which it was collected. 'Top rotifers' refers to the percentage of animals present on the surface of the standard medium in the Petri dish (Suppl. Fig. 2). In cases where the Ca^{2+}-free medium was applied, the missing salt content was compensated by sodium chloride.

The RIC production for MS purposes was performed using *E. dilatata* species under similar conditions to biofilm formation, except the animals were not fed. Due to their larger size, females were separated from males using filtration (using a plastic web) at the population level. Males intended for the same purpose were individually isolated into a water droplet (0.2 mL) using a micropipette. Once the desired number of animals (50 entities/drop) was reached, they were rinsed in additional droplets and monitored. Both genders were treated with metal epoxy beads, an inductor for biopolymer production, in a volume ratio (working dose 50 μg/mL), for 90 min. Removal of the animals (female population by a plastic web; males individually by a pipette) was followed by the collection of RIC, which was separated by magnetic fixation. The RIC isolation and storage (frozen) were carried out according to a previously described protocol [18].

To quantitatively compare the biopolymer production capacity of both genders, namely the quantity of RIC web, females were also used in small volumes, similar to males: $n = 12$ well/gender (20 ± 1 rotifer/well for 90 min; 24-well plate); the final (working) volume per well was 1 mL of standard medium. A mathematical formula published earlier [20] was applied to the current measurements. The biopolymer-producing capacity index is a relative unit: $BPC_i = A/B*C/D*E/F*G$; A: the average size of conglomerates (μm^2); B: average diameter of inductors (μm); C: covered area by conglomerates (%); D: induction period (min); E: space area (cm^2); F: starting number of animals per area; G: most extended filament of conglomerate (mm).

In addition to measuring the BPC_i of the whole rotifer population in one sample, the genders were compared regarding individual performance. We measured the size and extent of RIC filaments created by each individual during the 90 min. The pattern drawn by RIC was measured from digital photographs to obtain the summarized RIC length (mm) per animal ($n = 30$ entities).

2.6. Gel electrophoresis and visualization

Examination of the protein profile of the rotifers and Rotimer-containing materials, followed by an independent analysis of the separated samples of these panels, is necessary to determine the possible main components of the biopolymer. The rotifer-specific exudate-coated epoxy-metal beads (1 mg) were incubated with 40 μL standard (1×) Laemmli buffer (2 % SDS, 60 mM Tris-Cl pH 6.8, 10 % Glycerol, 0.01 % Bromophenol Blue, 100 mM DTT with or without 5 % mercaptoethanol) at 95 °C for 5 min. Samples were separated on 10 % SDS polyacrylamide gel. Gels were stained with Coomassie Brilliant Blue solution (0,05 % Coomassie Brilliant Blue R250, 50 % methanol, 10 % acetic acid) for 1 h and then destained with a mix of 50 % methanol and 10 % acetic acid for 3times 30 min [43]. It was necessary to apply an adequate silver staining method for the male exudate examination due to the low number of males that could be collected and analyzed [44].

The gel electrophoresis results were presented in a typical montage, where the reference markers, instrumental, and image processing backgrounds were kept consistent. The high-resolution images of the gel were digitally synchronized to the white balance of the homogeneous background, as both the Coomassie and silver staining results were displayed in one picture. The representative figure serves only for the qualitative demonstration of band positions. Quantitative determination and comparison, which are not methodologically relevant, were not the objectives.

2.7. Antibody-based RIC inhibition assay

For the experimental investigation of the main components of Rotimer, antibody-based inhibition was used, adapting the previously described tubulin [45] and fibrin [46] polymerization inhibition methodology. The investigation of the effect of anti-tubulin (as aspecific control), anti-SSPO, and anti-14–3-3 polyclonal antibodies on RIC formation and BPC_i values started with a 10-min pretreatment of rotifer populations with these proteins. As a next step, it was continued with a 90-min incubation as describe above, using powdered Carmine crystals [18] as inductor particle for RIC formation. The use of previously applied epoxy-coated beads are inappropriate in this case, since these particles are designed to bind antibodies so that an aspecific bond could develop between the treatment material and the inductor. The antibodies were used in a 1:400 dilution, where the final dose of sodium azide was 0.77 μM, which, based on our previous measurements [6], has no toxic effect when applied acutely. Identical to the antibody treatments (where the azide was initially included in the sample), this chemical background was also used for the control measurements. The BPC_i was evaluated in a similar manner as above. The experimental conditions were the same for both *E. dilatata* and L. *bulla*.

2.8. Rotifer genome and transcriptome sequencing and analysis

In order to determine the possible main components of the biopolymer of the most efficient producer, *E. dilatata,* it is inevitable to determine and analyze its genome and transcriptome. The reference transcriptome of *E. dilatata* was generated by applying a combination of short-read Illumina NovaSeq 6000 (Illumina Inc., San Diego, CA, USA) and long-read PacBio Sequel IIe technologies [47]. Trinity pipeline [48] was run to assemble the reference transcriptome (# isoforms: 70502). A draft reference genome was also prepared (from two independent sequencings; total genome length: 477 Mb, # contigs: 6645, N50: 341109, GC%: 40.3) using the long-read sequencing method. Gene models were built using Augustus [49] by mapping the transcriptome to the genome in order to create external evidence and hints for ab initio gene prediction. The analysis resulted in 13,673 complete and 8078 intronless genes that were used to create a reference database for the MS analysis. A separate paper will publish a detailed description of the reference genome and transcriptome.

2.9. Mass spectrometry analysis of E. dilatata RIC

In order to evaluate the main components of Rotimer, it is necessary to decide the complete protein profile of the available rotifer-secreting exudates. Proteins isolated from the magnetic epoxy-metal beads were suspended in 20 μL of 50 mM ammonium bicarbonate buffer (pH 8.5) and reduced at 60 °C for 15 min using TCEP (Tris-2-carboxyethyl-phosphine; 1 μL of 100 mM) followed by blocking of the resulting free thiol groups using MMTS (methyl methanethiosulfonate) at room temperature for 15 min. Subsequent tryptic digestion ($n = 3$, triplicated) was performed at 37 °C for 4 h with sequencing grade trypsin (0.5 μg). Proteolysis was terminated by adding 2 μL of 10 % TFA. Magnetic beads were immobilized using a magnetic separator, and the supernatant was used for further analysis. One third of the peptide mixtures were injected into an Evosep One (Evosep) – Orbitrap Fusion Lumos Tribrid (Thermo Fisher Scientific) LC-MS/MS system [50]. Peptides were separated using the '30 samples per day' 44-min gradient followed by MS/MS analysis of the highest abundance multiply charged precursor ions in each instrument cycle (cycle time: 3 s). The MS1 and HCD (higher energy collisional activation) data were acquired with high resolution in the Orbitrap analyzer. Proteins were identified using the Protein Prospector search engine using the Swiss-Prot and the reference database created from the genomics data.

2.10. Theoretical selection of the predicted Rotimer components

In the case of the Rotimer analysis, a complete set of RIC-peptides detected by the MS from the male samples was initially compared with a global protein database to select the non-specific (non-rotifer)

targets. Next, the endemic *E. dilatata* protein database (Suppl. Raw database) was applied, created from their annotated genome and transcriptome, to identify rotifer-specific molecule targets. The primary selection criteria for identifying Rotimer-specific proteins was to have at least five unique peptides representing the protein in the male samples; the number of unique peptides and the resulting protein sequence coverage should be in the right proportion after normalization. Then, the resulting 83 target sequences were further narrowed down using the female datasets. Finally, the intra- and extracellular localization of the selected 23 proteins was analyzed by DeepLoc2.0 [51] and BUSCA [52]. Because the Rotimer accumulates externally, the cytoplasmic, mitochondrial, and nuclear proteins were excluded from further analysis, and only proteins showing extracellular prediction were retained. The bands (117 excised samples) obtained during gel electrophoresis from the rotifer-specific samples (whole animals, biofilm, and biopolymer) were also examined and applied as confirmatory data. The basic experiential properties of Rotimer (e.g., Ca^{2+}-dependency of the producing process, viscoelasticity, rapid formation) are common in all investigated rotifer species by our laboratory; therefore, the presence of predicted targets in published annotated rotifer genomes and proteomes [53,54] was an essential criterion during final selection. Due to this stringent restriction, four proteins (rf 1 ctg.000390F.g65781.t1; rf 1 ctg.000595F.g78629.t1; rf 1 ctg.000009F.g13179.t1; and rf 1 ctg.000013F.g14825.t1) in two categoric types were selected as the predicted main and associative constituents of Rotimer. For structure prediction of the predicted main component of Rotimer, the Alphafold2 colab [55], a machine learning-supported method was applied [56]. The predicted structure models were visualized by UCSF Chimera [57].

2.11. Statistics

The multi-omics analysis is independent of the experiential measurements, so it is detailed in the given methodological section. Statistical analysis of the empirical experiments was made with GraphPad Prism 7.0b software (GraphPad Software Inc., La Jolla, CA) using one-way ANOVA with Bonferroni post hoc test. The error bars show the standard error of the mean (SEM). In parallel with the above statistics, a 99 % confidence level (1-alpha = 0.01) was applied, and a 1 % error was handled. The homogeneity of variances was controlled; however, the empirical data were parametric, and they were found optimal for one-way ANOVA with Bonferroni post hoc test. The significance variable levels are as follows: $p* \leq 0.05$; $p**,## \leq 0.01$; $p*** \leq 0.001$ and $p**** \leq 0.0001$. The figure legends independently detail the statistical comparisons and correlations between the affected groups.

3. Results and discussion

One of the emerging research areas regarding rotifers is the study of exogenous biopolymers they produce, aiming to investigate their properties, roles, regulation, and potential applications. This ability of rotifers has already been published in previous studies [18–20]; however, their facilitating involvement in higher-order biofilm formation is a novel finding. The forms of appearance of biopolymers can be liquid, gel, or solid-state, found in freshwater and marine environments, with proteins being the predominant component [58,59], such as in Rotimer.

The primary experimental objective of this study was to explore the functional properties and predicted composition of the exogenous biopolymer produced by rotifers, especially focusing on the monogonont *E. dilatata*. Understanding the role played by rotifers in their natural environment and isolating a sufficient quantity of biologically relevant material (exudate) were given high priority in this study. Therefore, the production, isolation, preparation, and examination of any rotifer-derived samples that may potentially contribute to determining the composition of Rotimer were emphasized.

3.1. Rotimer-related biofilm formation in rotifer cultures

A novel observation was presented during the methodological optimization in Rotimer investigation: the formation of a rotifer-specific biopolymer-induced biofilm on their culture medium surface. This complex and intriguing organic mass creation by prokaryotes is initiated, based, and mixed by the rotifer-specific RIC formation. In this case, it occurs through particle-induced network formation, followed by rafting and layering on the medium surface (Fig. 1A-D). The rotifers actively engage in feeding and reproduction in the lower region of the formed biofilm layer.

All four investigated rotifer species (Fig. 1: *E. dilatata*, **A**; *L. bulla*, **B**; *P. acuticornis*, **C**; *A. vaga*, **D**) can generate the above-mentioned biofilm. The Rotimer of these micro-metazoans is certainly involved in forming the water surface layer, as in a semi-sterile environment, perhaps with the bacteria from the environment or with endosymbionts released from dead animals. During the microscopic monitoring of this organic system, active bacteria were also visible in the samples. Yet, their independent examination or identification was out of the scope of the current study. The RIC, created by rotifers, can also be an initiating structure and component, resulting in a concentration process of organic debris, and is likely a source of nutrients for the prokaryotic populations, resulting in biofilm creation by these external polymeric substances. No conglomerate formation was observed for non-ionic polystyrene particles (10 μm Ø).

The first step of the biofilm creation process is the migration of organisms to the water surface, spanning even several millimeters (Suppl. Fig. 2). In our artificial microecological system, this migration can be easily monitored optically. In the present circumstances, the biofilm formation did not start without rotifer individuals floating to the surface. If they were fed with completely settling granulates larger than 100 μm in diameter, the entire population remained on the bottom of the culture. In the case of providing granulates with a maximum diameter of 50 μm, only island-like rafts appeared initially, which then gradually connected over a few days, forming a cohesive membranous layer (Suppl. Fig. 2A). This product is a solid material with a white appearance that can be easily collected with a spatula (Suppl. Fig. 2B). Three fundamental requirements for its formation have been identified: low (30 %) humidity (Fig. 1E), particle-type stimulating inductors (e.g., food coagulates) [18], and the presence of Ca^{2+} [19]. The need for the presence of this metal cation is similar to the case of Rotimer secretion and, thus, RIC production. The 'biofilm formation rate' is a formula encompassing two independent parameters, developed to compare different rotifer species and relevant conditions regarding biomaterial layer formation on the water surface. Lower environmental humidity leads to more intensive migration of rotifers towards the water surface and more efficient formation of the surface-closing layer. At 30 % humidity, the monogononts were the most active among the examined species, with *E. dilatata* being the most active, facilitating twice as large biofilm amount as L. *bulla* and four times more than the bdelloids. The triggering factor for this process is presumed to be the perception of animals related to the surface evaporation of their medium (which is inversely proportional to air humidity) and the increase in local salt concentration. This biological system creates a surface-sealing biofilm, as a defense mechanism against these habitat-threatening factors. Underneath the biofilm, they engage in the same activities, such as egg-laying, feeding, and animal interactions, as the bottom-dwelling individuals on the underlaid. In conclusion, it can be stated that biofilm formation depends on the intensity of Rotimer secretion. When the rotifers were tested under completely identical conditions (e.g., medium, food, number of individuals, or atmosphere), the product differed for each species. This evolutionary and ecologically complex process was described for the first time related to rotifers.
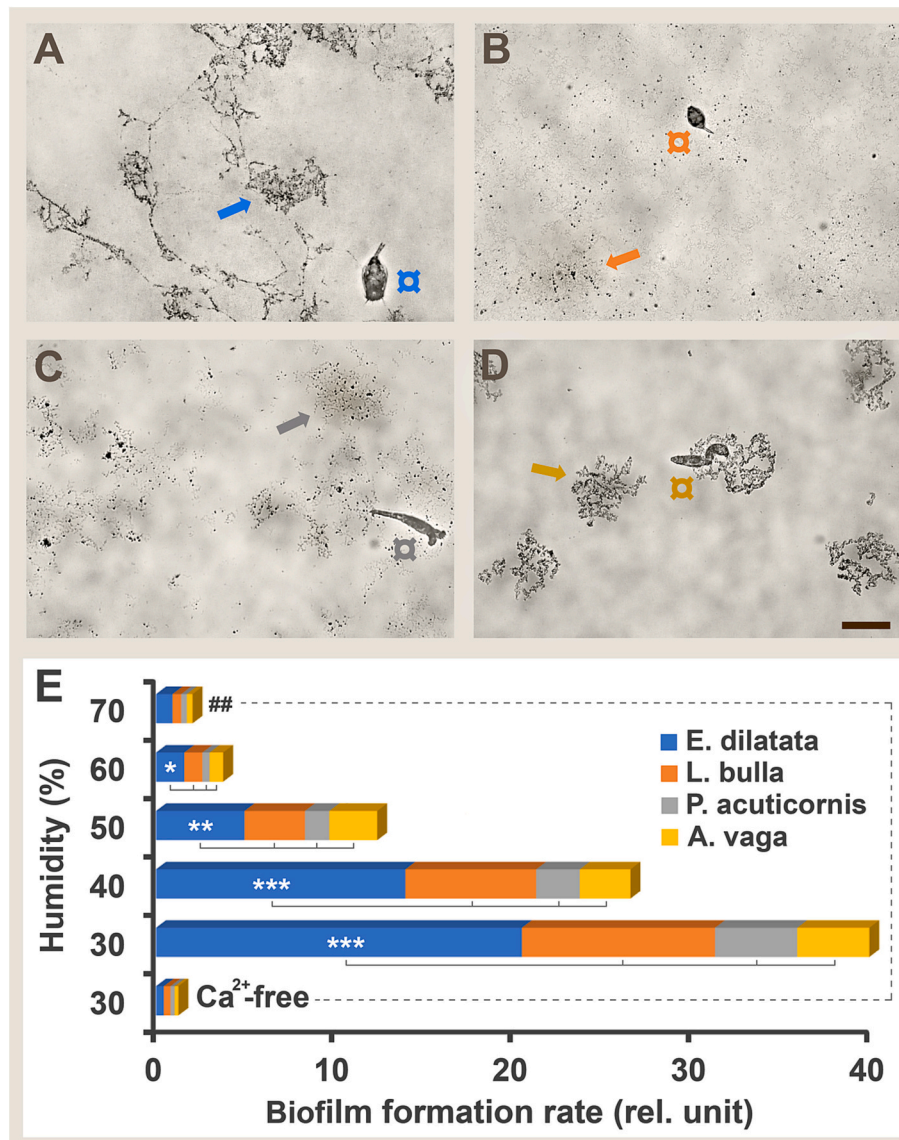
Fig. 1. Humidity-dependent biofilm formation by rotifers. The biofilm initiatives (rafts) on medium surface of *Euchlanis dilatata* (A), *Lecane bulla* (B), *Philodina acuticornis* (C) and *Adineta vaga* (D) species (scale bar is 0.2 mm) and the effect of humidity and Ca^{2+} to biofilm formation rate (G) are presented (the arrow shows the localization of a representative raft on the medium surface; ¤, individual rotifer from the relevant species). One-way ANOVA with Bonferroni post hoc test was used for statistical analysis, the levels of significance are $p* \leq 0.05$; $p**,\# \leq 0.01$ and $p*** \leq 0.001$. (*, significant difference of the *E. dilatata* from the rest of the rotifer species in the same conditions; #, significant difference of the rotifers from their own species in the Ca^{2+}-free groups).

3.2. Rotimer secreting and conglomerate forming ability of E. dilatata males

The most intense Rotimer secretion capability and the highest yield of biofilm and RIC formation determined the selection and application of *E. dilatata* and their exudate forms for further examination (Fig. 2). Additionally, this species is dimorphic having female (Fig. 2A) and male (Fig. 2B) individuals with characteristic differences in size, anatomy, feeding, and lifespan [60]. The females are larger than their male counterparts, having about ten days lifespan. The dwarf males have degenerative and inactive digestive systems [61] and have a maximum longevity of 2 days.

Similarly to females, the freshly hatched male individuals are also capable of Rotimer secretion (Fig. 2C) and thus, RIC formation. The biopolymer substrate is present in animals from birth, meaning its precursor develops within the body during individual development. Another difference between the sexes is that the epoxy-metal beads can adhere to the exoskeleton of males (Fig. 2C), indicating the presence of

some adhesive layer on the body surface of rotifers. Similarly, particles adhering to their surface were also observed in bdelloids during the formation of their conglomerate [20]. Monogonont females (*E. dilatata* and L. bulla) do not exhibit such external attachment, regardless of the age of the animal. Nonetheless, females also cannot organize the inductor particles into fiber form with a deficient Ca^{2+} presence (<1 mg/L),; moreover, the RIC can even stick hard to their bodies or cilia.

The existence of Rotimer produced by males has been detected and visualized for the first time, confirming that both sexes are capable of biopolymer production. The random macro-structure, formation kinetics, and phenotype of the RIC web (Fig. 2D) are similar in fundamental characteristics to the formation created by females previously described [19], having only differences in the amount of produced RIC.

The formation of direct physical contact between the sexes [60], which lasted for several minutes, was frequently observed in laboratory cultures, and likely corresponded to the act of sexual reproduction. Interestingly, during the manual collection of males (using a micropipette), they often pulled the females along with them, not through direct
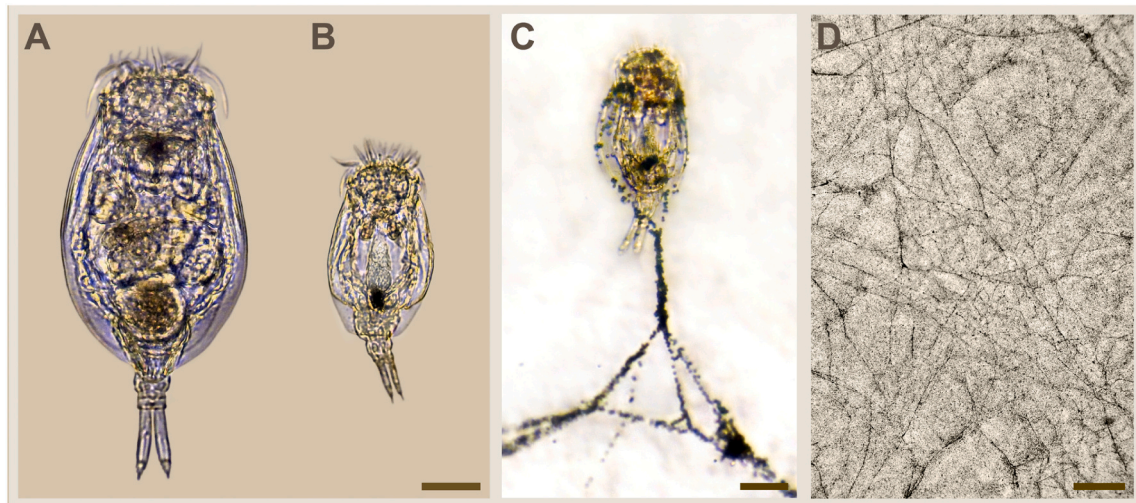
Fig. 2. Presentation of *Euchlanis dilatata* characteristics. Anatomical differences between female (A) and male (B) rotifers; Rotimer-Inductor Conglomerate (RIC) formation by male rotifers and the body surface collected (attached) epoxy-metal beads (C); RIC-web formed by selected male rotifer population (D). The scale bar is 50 μm (A-C) and 1 mm (D).

contact but at a distance of a few microns. This phenomenon suggests a thread-linked state between them in their microenvironment. Based on empirical observations, it is hypothesized that Rotimer plays a direct role in the spatial orientation of rotifers (e.g., newborn individuals stay near the empty egg capsules or regularly return there within the first hour), interactions between individuals, mutual recognition, and
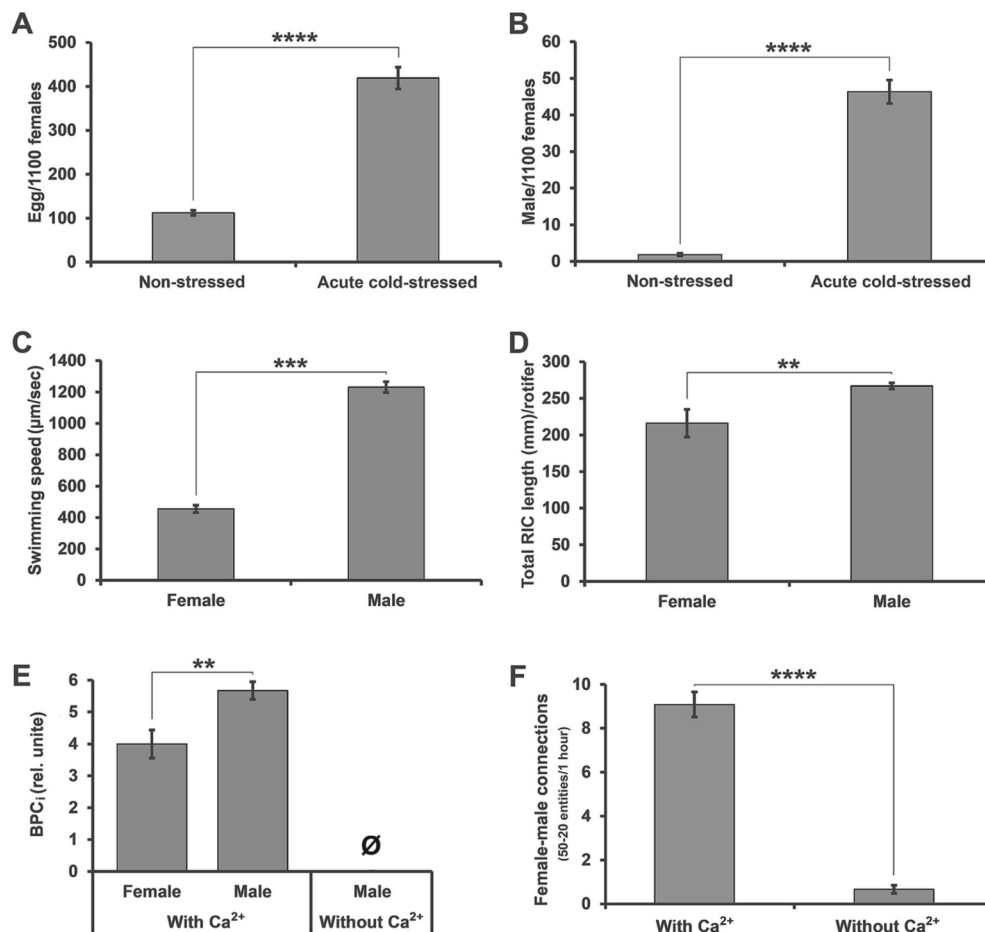

Fig. 3. Phenotypical and experiential characteristics of female and male relations and differences in *Euchlanis dilatata* species. The egg (A) and male (B) numbers, swimming speed of genders (C), total length of rotimer-inductor conglomerate (RIC) per individuals (D), biopolymer-producing capacity index (BPC$_i$) of genders (E) with and without Ca^{2+} in environment, and physical connections between genders (F) were presented. 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 and p **** $\leq$ 0.0001. (*, significant difference from the other groups).

communication.

3.3. Culturing and investigating E. dilatata males in relation to Rotimer

Given the availability of the new Rotimer patterns offered by males, a specific objective was established to analyze them. The number of eggs laid in an average population (Fig. 3A) and the proportion of male individuals born from those eggs (Fig. 3B) were insufficient for RIC production purposes. To stimulate reproductive capacity, acute cold-stress was applied. In the stimulated colonies, the number of deposited eggs significantly increased after one day (almost 4-fold increase; Fig. 3A), and similarly, the proportion of male offsprings was also higher than that of the controls after one day (25-fold increase; Fig. 3B). Neonates hatched after one day, typically, first the female individuals (after 20–22 h) followed by the males (after 24–26 h). Working with the achieved population size, the entities were collected individually, washed, and stimulated with inductor particles, resulting in a measurable quantity of exudate formation.

Rotifers produce RIC fibers at a speed corresponding to their swimming speed, a relatively fast process in micro-metazoans. This fact was already known from previous studies [19]; however, the observation itself was interesting, because the ability of males moving [60] and forming the conglomerate more than twice as fast (1.2 mm/s) as females (Fig. 3C) was monitored. With such BPC kinetics, it can be assumed that the major component of the biopolymer may be an evolutionarily conserved and biochemically abundant substance that precipitates in an aqueous medium, likely under the influence of Ca^{2+}. Furthermore, individual male specimen formed longer RIC fibers (overall length: 267 ± 4 mm) independently, compared to females (overall length: 216 ± 19 mm) (Fig. 3D). Despite the detected width [18] of the formed Rotimer fibrils (average diameter: 30 nm), and drawing on the average dimensions of the internal organs of rotifers, the predicted volume of biopolymer inside the animals is calculated to be relatively small (170–190 μm^3/entity). Thus, it can be assumed that these animals can achieve high functionality even with a small amount of material. Regardless of the organ in which this biopolymer is produced and stored (e.g., salivary glands or retrocerebral organ), the volume of its pro form occupies only a maximum of 2–4 % of the respective organ's capacity. On the population level, a significant difference (1.4-fold) in favor of males was observed in the biopolymer-producing capacity (Fig. 3E). The relevant formula, considering numerous factors, has been applied on several occasions [20] as it represents the exudate production capacity of rotifers with sufficient biological relevance. The previously demonstrated Ca^{2+} dependence of Rotimer secretion by females [19] was also confirmed in males. Similarly, the number of direct interactions between the sexes depended on the presence or absence of this metal ion, which may indicate the necessity of their biopolymer in sexual reproduction. It is essential to mention that the formation of Rotimer in the external space is polarity-dependent since neither of the two sexes was capable of RIC formation when induced by apolar polystyrene beads (Suppl. Fig. 3).

The experiential monitoring of interactions between the sexes, and the additional results obtained, all support the conclusion that (aside of nutrition and reproduction) biopolymer formation is one of the essential central roles and phenotypic characteristics of rotifers.

3.4. Representative protein panel of rotifer and Rotimer containing samples

For determining the special Rotimer components, primarily multi-omics methods were applied, starting from direct biopolymer-containing samples; moreover, these sources were also examined from a biochemical perspective. The reproducibility and consistency of the samples containing Rotimer were pre-monitored and verified using gel electrophoresis, with multiple extracts from different measurements presented in the montage (Fig. 4). These extracts are only shown for representative and illustrative purposes and are not quantitatively
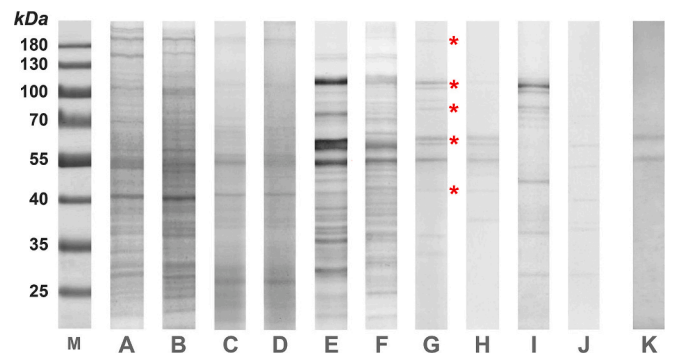

Fig. 4. Qualitative presentation of rotifer- and their exudates-related protein profile. Total protein of species: *Euchlanis dilatata* (A), *Lecane bulla* (B), *Philodina acuticornis* (C), and *Adineta vaga* (D); biofilm of species: *E. dilatata* (E) and *L. bulla* (F); rotimer-inductor conglomerate (RIC) proteins of females: *E. dilatata* (G; SCO-spondin hits by mass spectrometry analysis, marked with red star) and *L. bulla* (H); RIC-containing proteins isolated with mercaptoethanol-free sample buffer: *E. dilatata* (I) and *L. bulla* (J); RIC-containing proteins of males (K). The marker (M) indicated a range between 25 and 180 kDa.

characterized.

After optimizing the isolation procedures, the reproducibility rate of protein-containing rotifer samples was above 95 %. The total protein extracts of the rotifer populations (Fig. 4: *E. dilatata*, A; *L. bulla*, B; *P. acuticornis*, C; *A. vaga*, D) showed high similarities around 40 and 55 kDa. The biofilm samples of the two monogonont species (Fig. 4: *E. dilatata*, E; *L. bulla*, F) exhibited different profiles compared to the comprehensive analysis. However, the enrichment of proteins around 55–65 and 110 kDa was observed. It is assumed that these samples should contain the target molecules; however, the patterns were too divergent for precise identification.

In the case of complex composition cultures, the biofilm samples were expected to be highly contaminated with food and bacterial proteins (endosymbionts and exotypes in a semi-sterile environment).

The RIC complexes were considered cleaner samples than the biofilm, with a higher proportion of biopolymer content (Fig. 4: *E. dilatata*, G; *L. bulla*, H). While the biofilm originated from a highly complex, multi-membered, and diverse environment, RIC formation is considered a more purified system with a selected and washed female population (thousands of individuals per case), where the samples are very slowly contaminated with food and environmental bacterial proteins. In this measurement, RIC-proteins appeared explicitly around a similar range as that of the biofilm, particularly in the case of *E. dilatata* (Fig. 4G). Furthermore, it is interesting to note that the massive sample formed in a co-culture (prokaryote and eukaryote) also showed the same main band positions as the Rotimer exudate, produced in an isolated and controlled environment, thus pointing to the role of rotifer biopolymers in biofilm formation.

Protein extracts, using classical and mercaptoethanol-free Laemmli buffer protein isolation methods, were compared to test for the presence of disulfide bonds in the Rotimer. In this case, a completely different pattern emerged compared to the aforementioned (Fig. 4: *E. dilatata*, I; *L. bulla*, J). While the band around 110 kDa remained, the previously observed region around 55–65 kDa disappeared in the mercaptoethanol-free conditions. The changes in protein size patterns between different preparation processes and the later discovered and presented (using omics methods) high molecular weight values collectively suggest that proteins may undergo hydrolysis and fragmentation during production, isolation, and the preparation for gel electrophoresis.

Although the purity of rotifer RIC samples of females is superior to the biofilm, there is still too much contamination to identify the target molecules. The underlying explanation for this phenomenon may be that rotifers produce their exudates on the inductor (epoxy-metal beads), physically consume these particles, and then excrete them. The

induction cycle is several times longer (90 min) than the digestion cycle (20–30 min), resulting in approximately one-third of the RIC samples being contaminated with materials from the intracellular and digestive system. For these less-than-optimal reasons, obtaining RIC samples with as little non-specific material as possible was necessary.

Realizing that males of *E. dilatata* also produce biopolymer and form RIC in response to induction provided an opportunity to extract cleaner exudates. Unfortunately, the proportion of males appearing in the natural environment is low. However, the temperature-based cold-stress stimulation method to increase their numbers proved to be effective. Using individually selected, thoroughly washed and homogeneous populations of males (Suppl. Fig. 4), RIC could be produced with the cleanest exudate content (Fig. 4K). The individuals do not eat due to their degenerate digestive system, making the inductor particles non-contaminated with endogenous proteins. The bands around 55–60 kDa were also present here. Despite of using a highly sensitive silver staining method, it cannot be excluded that other proteins were also present in the gel due to the low amount of exudates of males (secreted by a few hundred individuals).

Although protein separation in the gel was primarily applied to verify the reproducibility of the samples (rotifers, biofilm, and biopolymer), a portion of the excised and isolated bands (Fig. 4**E-H** and **K**) was further examined using MS. Similarly to samples prepared with standard direct enzymatic digestion used in proteomic methods, gel digestion delivered specific peptide sequences that served for the identification of the presumed main components of the examined biopolymer.

3.5. Multi-omics analysis of E. dilatata and its exudates

Previous studies [18–20] have already established that exogenic exudate-type biopolymer production is a common ability of rotifers, secreted by both monogononts and bdelloids. In the examined species these exudates showed the same experiential properties, such as web structure, formation kinetics, elasticity, tensile strength, size ratios, physico-chemical and biochemical properties. Based on the universal production of exogenic exudate-type biopolymer, it can be assumed that the specific major components are also phylogenetically conserved, abundant, general, and almost identical. After the primary (technical and physiological) selection of potential target proteins, the presence or absence of candidates in the relevant genomic and proteomic database [53,54] of rotifer species was considered in the final decisions.

Two independent genomes and two different transcriptome sequencing in the case of *E. dilatata*, together with the confirmatory L. *bulla* genome analysis data and other six (protein blast NCBI GenBank identification numbers: GCA_023231475.1; GCA_905250095.1; GCA_905250115.1; GCA_905273325.1; GCA_905331475.1; GCA_9053 32065.1) annotated rotifer genomes [53,54] served as the basis for the MS-related bioinformatic analysis. The RIC samples produced by both sexes of *E. dilatata* were subjected to proteomic analysis. The enzymatic digestion and MS peptide sequences obtained, along with genomics and transcriptomics databases, were used to match and determine the potential biopolymer-specific and non-specific proteins present in the rotifer exudate. First, a minimum of five peptide sequence hits were considered in the male samples. Additionally, the extracellular profile, including the external environment, was considered. Ensuring that the identified hits were present in the female samples was also essential. Interestingly, the RIC products of females resulted in fewer peptide hits during the MS analysis compared to their counterparts. This discrepancy can be attributed to a higher degree of contamination affecting the females and subsequent molecular competition occurring on the surface of the inductor particles (metal-epoxy beads with high polarity and adhesion properties). As a result, the conglomerates of Rotimer primarily formed by males served as the initial basis for the theoretical selection of target proteins. It is difficult to define whether the Rotimer consists of several functional or only one specific component. In the latter case, it

only encloses itself and thereby traps the proteins from its molecular environment during precipitation (at a speed of 1.2 mm/s). The formation process of the exudate is Ca^{2+}-dependent, without assuring that the main component would be the same. This metal ion is needed in situ and acutely for the rotifers; however, it may be needed physiologically for releasing the nonsolid precursor material into the external space, or it may be needed for molecular precipitation during the solidification of the biopolymer.

Based on the available multi-omics and experimental data, the most probable main components of the Rotimer are members of the evolutionary conserved SCO-Spondin (subcommissural organ-spondin; SSPO) protein family (Fig. 5A), associated with obligatory eukaryote-specific 14–3-3 protein (Fig. 5B). These two molecule families show opposite specificity in terms of their occurrence in nature. The main component, SCO-Spondin, is exclusively identified in rotifers (R-SSPO/1, rf 1 ctg.000390F.g65781.t1, MW: 321850, total/max score: 2709/2219; **Suppl. Raw database**), and the other two SSPO proteins (R-SSPO/2, rf 1 ctg.000595F.g78629.t1, MW: 445537; or R-SSPO/3, rf 1 ctg.000009F. g13179.t1, MW: 581207) are mostly present in rotifers, along with some marine species (*Lytechinus pictus*, *Anneissia japonica*, *Ostrea edulis* or *Crassostrea virginica*; based on NCBI and UniProt blast data). In the MS analysis of *E. dilatata*-RIC, the R-SSPO/1 had the highest number of unique (51) and total (88) peptide hits, with even distribution along the annotated sequence, among all identified proteins. In contrast to R-SSPO, the sequence of 14–3-3 protein (rf 1 ctg.000013F.g14825.t1; MW: 28171) is universal and uniform (up to 98 %), even among species that are phylogenetically distant from one another.

In addition to the results listed above, there is also an uncharacterized extracellular protein (rf 1 ctg.000064F.g30789.t1), which contains domains similar to R-SSPOs, except for the presence of C-type lectin domains. Nevertheless, its general occurrence in rotifers is low (BLAST bit-score: 65) and based on the regularity of the general presence of Rotimer, this result cannot be considered a possible main component, perhaps only an *E. dilatata*-specific Rotimer-associated protein.

The SSPO proteins are mostly large (even 540 kDa) molecules, with very high variability in isoform versions [62,63]. These glycoproteins of the thrombospondin family were primarily detected in the nervous system of vertebrates [64]. The most common sequence patterns of SSPO-specific von Willebrand factor (VWF) are the VWF-type D domain (VWFD), low density lipoprotein (LDL)-receptor class A (LDLRA2) domain profile, Epidermal Growth Factor-3 (EGF-3)-like domain, C-terminal cystine knot domain (CTCK), trypsin inhibitor-like domain (TIL), and Elastin microfibril interface domain (EMI) [65,66]. Several disulfide bridges are also present within the domains. Variation of amino acid sequence-specific motifs or domains during protein formation is a known phenomenon in the living world.

The SSPOs had a wide range of functions (e.g., neuronal development, modulation of aggregations, ependymal differentiation, and fibrillization). It should be highlighted that it participates in forming Reissner's fiber (RF) and has Ca^{2+} binding ability by EGF-3-like domain [62,67]. The soluble SSPO can be localized intracellularly, and extra-cellularly in a polymerized form. This dual and, at the same time, dynamic occurrence is significant in the cerebrospinal fluid (CSF) of vertebrates. Alternative splicing and proteolytic cleavage can produce several isoforms of these proteins, with a wide range of sizes. Among the diverse domains, VWFD, CTCK, TIL, and EMI play a role in forming the RF; of these, the first two are primarily mentioned. This special polymerization means networked and relatively (even mm) long complexes with different macromolecular levels (flocculent, fibrils, pre-RF, and RF) and an especially adhesive surface [64]. It is also able to bind abnormal and toxic aggregates, such as amyloid-beta precursor protein derivatives, thereby purifying the CSF. Similarly, with the formation of RIC, rotifers can trap the macromolecular particles in their environment and extract them from the water as a kind of flocculation, thereby keeping it clean. The RF formation can take from a few hours to days in
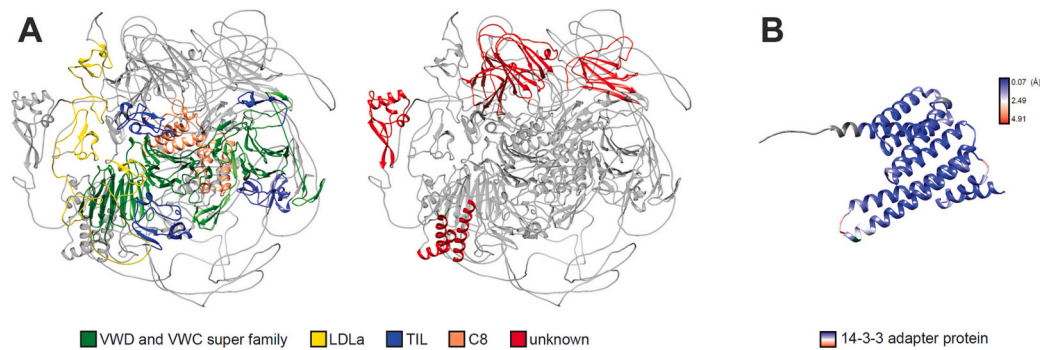
Fig. 5. AlphaFold structure prediction of R-SSPO/1 and 14–3-3 adaptor proteins. Two representations of the predicted spatial conformation of the R-SSPO/1 (rf 1 ctg.000390F.g65781.t1) protein: predicted structure colored according to conserved domains identified by CD-search and predicted structure highlighting further globular motifs are highlighted (red) that were predicted with acceptable confidence (A). Predicted model of 14–3-3 adaptor protein (rf 1 ctg.000013F.g14825.t1) with a highly conservative structure (B).

the CSF [68]. Although the EGF-3-like domain has Ca^{2+} binding capacity and is part of SSPO proteins, RF formation specifically has not yet been linked directly to Ca^{2+}. Except for these two parameters (in rotifers, the production of solid exudate takes place in seconds and depends on Ca^{2+}), all other parallel characteristics can be drawn between the composition and formation of RF and Rotimer. The environmental conditions explain the difference in the formation kinetics between the two systems. The RF is created in a hyper-regulated and closed system, while Rotimer is secreted in an open-spaced aquatic medium with low osmolarity.

Representative and exclusively qualitative gel montage (Fig. 4) was applied to control the reproducibility of rotifer cultures, biofilm, and Rotimer exudate. Despite the results of the MS analysis, the protein bands were not the primary aspect of the Rotimer identification; they provided confirmatory data. The R-SSPO proteins were present in 5 of the 117 excised *E. dilatata* samples (Fig. 4G; around 45, 65, 85, 110, and 190 kDa; labeled with red asterisks). The reason that the target molecules were present in several bands could be explained by the variation of isoforms, or with fragility of proteins due to their large size during preparation, such as the breaking up of disulfide bridges or chemical hydrolysis-based fragmentation. The following domains were predicted to be present in the R-SSPO proteins found: VWFD, VWFC, EGF-3-like, LDLRA2 profile, TIL, Extracellular matrix glycoprotein related, serine protease inhibitor-like superfamily, VWF/SSPO/Zonadhesin-like, C8, laminin, semaphorin-5 A isoform X1, and thrombospondin type-1 repeat superfamily (source: www.ebi.ac.uk/interpro and prosite.expasy.org/).

The representatives of this protein family are presumed to constitute the composition and external formation of Rotimer, and some RF-special properties (e.g., common domain profiles, fibril formation, and Ca^{2+}-dependent process) are similar to the currently explored exudate. However, the complex (Rotimer) formed by these molecules and its predicted formation mechanism (within seconds) are considered novelties. In the future, it would be worth exploring the mechanism by which the presumed liquid or gel-like preform of Rotimer transitions into a solid viscoelastic state, be a relevant in vivo model for CSF-localized RF in the development of the embryonal central nervous system [64]. Performing in situ studies in humans or vertebrate animal models is not readily feasible. However, rotifers and their environment can provide solutions for understanding the critical elements of the formation mechanism of extracellular and external-based filaments.

The ability for polymerization and the complex structure of SSPO may be a possible connection and explanation for the previously described [20,69] specific effects of Rotimer related to the inactivation (in vivo) of human-type neurotoxic aggregates (beta-amyloids, alpha-synucleins or prions). Moreover, it has anti- and disaggregation properties (in vitro) against beta-amyloid 1–42. The special amino acid sequences of SSPO proteins may be suitable for a drug development information base [70,71] due to their widely occurring, yet central

properties.

The second type of molecule, identified and predicted to play a role in the development of Rotimer, was the 14–3-3 protein. The extremely conserved, exclusively eukaryote-specific and both cytoplasmic and extracellularly [72,73] present adaptor 14–3-3 protein is a universal and multifunctional molecule with thousands [74] of interaction partners and regulatory effects in cellular processes [75]. It can indirectly interact with specific client targets or functionally with different sequential motifs, such as VWF [76] domains of its molecular targets. The core region of this protein binds the multitude of proteins; however, the termini are divergent. Its overall biological role in the extracellular space is still unknown, but its ability to bind Ca^{2+} [77] may be interesting from the point of view of Rotimer secretion. The identified 14–3-3 protein in this study has the following functional motifs: 14–3-3 protein signature 1 and 2, zeta signature, and gamma 1. Numerous connections of this regulatory protein were shown concerning human diseases, primarily related to the central nervous system, such as synaptic plasticity impairment [78], prion disease [79,80], and Alzheimer's disease [81,82]. In these cases, their application as a disease marker was also possible. We believe that this protein may contribute, directly or indirectly, to R-SSPO-based Rotimer formation. Based on the information in academic literature, it is not core component of Rotimer according to its profile; however, it may be a functionally associative partner of R-SSPOs.

Based on our current knowledge, no direct relationship between SSPO and 14–3-3 proteins has been described. There is an indirect similarity between them, because both molecules play a central role in the regulation of the vertebrate CSF, bind Ca^{2+}, are phylogenetically conserved, and bind to other molecules in great variations. In the case of SSPO, the modification ability of the 14–3-3 protein on the client targets can be regulatory, adaptor, or have activation function, leading to the formation of the Rotimer. It can be assumed that in the future, it will be possible to find a connection or to demonstrate a direct molecular interaction between these agents of interest in rotifer research. In that case, this complex can still be created within the body of animals, preparing for precipitation, polymerization, or at least for volume growth in the presence of calcium in an aqueous medium.

Among the cytoplasmic proteins, tubulins (rf 1 ctg.000102F.g43596. t1; rf 1 ctg.000121F.g39954.t1) were present to the greatest extent in the various samples. These proteins may primarily originate from damage to the cilia of rotifers or other parts of the body (e.g., the digestive system). The common family relations of the FtsZ prokaryotic proteins with tubulins have been described, as well as their ability for in vitro precipitation in the presence of Ca^{2+} [83]. Their characteristic profile is similar to the Rotimer (e.g., Ca^{2+}-dependent precipitation), but due to FtsZ cytoplasmic localization, it cannot be relevant in the present case. The role of these intracellular protein families in the formation of Rotimer cannot be ruled out, but despite this, they cannot be considered

main component candidates. Although direct binding of SSPO proteins to tubulins has been described [84], this molecular relationship in different extracellular spaces does not seem to explain the formation of external exudate of rotifers.

3.6. Inhibited RIC formation by SSPO-specific antibody

Exploratory multi-omics studies have pointed to the possible main components of Rotimer applying protein selection and prediction, based on genomics and MS results. Confirmatory experimental measurements were also carried out (Fig. 6), where the ability of rotifers to form exudates, realized in RIC, was investigated, using specific antibodies (anti-tubulin, anti-SSPO, and anti-14–3-3). BPC_i was applied as a marker parameter for this process. The anti-tubulin antibody was chosen as a non-specific control (the tubulin was present in MS-analyzed RIC samples but was not predicted as a main component of the Rotimer). After adding this ingredient to the medium of animals, it proved ineffective during their conglomerate formation. In contrast, the anti-SSPO antibodies caused a significant and biologically relevant decrease in the rate of RIC formation in both applied monogonont species, especially in *L. bulla*. Although the anti-14–3-3 antibody showed an antagonistic effect in forming the biopolymer-based network, its effect was not significant. In addition to being predicted as one of the main components, the possible role of this adapter protein in the structure of externally developed Rotimer requires further investigations. Likewise, these target rotifer-type proteins (tubulin, SSPO, and 14–3-3) contain specific epitopes from the corresponding peptide sequences (published by the manufacturers) used for producing their relevant antibodies and warrant further study.

The multi-omics analyses were performed on the most effective rotifer species in biopolymer production, i.e., *E. dilatata*; however, to determine whether the role of SSPO in the formation of Rotimer is species-specific or more general, we also looked at *L. bulla* in the current experiment. The BPC_i value of *L. bulla* is naturally lower than that of *E. dilatata* [20]. Based on the results, we believe SSPO is not the defining component of the Rotimer for only one rotifer species. It was impossible to test the antibodies acutely in situ on the bdelloid species because they tear their filaments apart after about 15 min [20], so the amount of RIC produced is insufficient to detect biologically relevant differences. Polymerization processes specifically inhibited by antibodies are known in several testing procedures [45,46], so these experimental results obtained in our case also lead to the conclusion that SSPO proteins can be decisive and necessary components of rotifer-specific biopolymers.

3.7. Conceptual view of Rotimer and its predicted components

Through chemical and biochemical processes and physiological
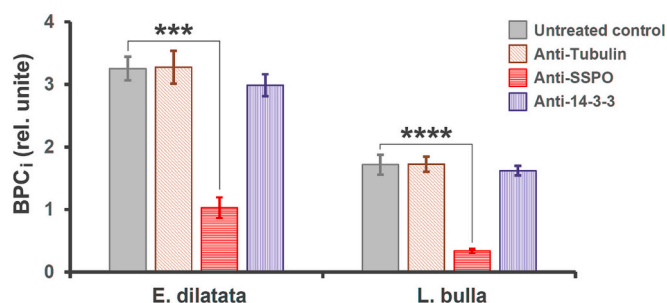

Fig. 6. The effect of acutely applied antibodies on the biopolymer-producing capacity index (BPC_i) of monogonont rotifers. The effect of anti-tubulin, anti-SSPO, and anti-14–3-3 polyclonal antibodies on the Rotimer-based conglomerate web formation is presented. The error bars represent SEM. One-way ANOVA with Bonferroni post hoc test was used for statistical analysis, the levels of significance are p*** $\leq$ 0.001 and p**** $\leq$ 0.0001 (*, significant difference from the untreated control group).

relevancies, the R-SSPO, upon entering the external environment, along with Ca^{2+} and other associative proteins, can form a biopolymer with consistent phenotype and function. In the case of investigated rotifers, consistency would be achieved if a sufficient quantity of a specific biomaterial were produced based on the outlined biological principles and mechanisms. This concept may give an explanation for why and how almost every rotifer species can produce such adhesive, high tensile strength, and similarly multifunctional exudate. Genetic redundancy, mechanistic elasticity and variability at the protein level preserve the molecular profile; furthermore, a unified Ca^{2+} and polarity dependence is necessary to achieve this. Only the above-mentioned molecular attributes together can make this material universally produced in sufficient quantities within the world of rotifers.

After determining the possible main and associated protein components of Rotimer, the next question is the formation mechanism of this biopolymer in an aqueous environment. The assembly of similar and related gel-type biopolymers (e.g., mucins) is a very complex process, mainly multi-component and Ca^{2+}-dependent [85]. It is possible that, in addition to those identified, other components (e.g., proteins, sugars, metal ions) are also involved in the structure of the rotifer-specific exudate or its formation process. However, their clarification requires further investigations in the future and is not part of this study. Furthermore, it will be interesting to explore how the external formation of Rotimer depends on Ca^{2+}; nevertheless, the conglomerate network does not disintegrate under the influence of a high concentration of metal ion chelator (EDTA) [18]. The Ca^{2+} is probably essential for precipitation, but then, the stability of the solidified water-insoluble protein complex (in fiber and glue-like form) no longer requires its presence.

The relevant experiential data known so far (e.g., aqueous medium, cation dependence, proteolytic properties, tight formation kinetics, high tensile strength, and vital functionality) lead to the conclusion that Rotimer is stored in a liquid or gel-type preform in one of the organs (possibly in the salivary gland) of rotifers. Moreover, in the case of ciliary irritation and the presence of a polar surface, it reaches the external (outside the body) space, where it solidifies and polymerizes, presumably by precipitation. Our results and the theoretical parallel between RF and Rotimer are among the most likely explanations for the rapid external processes of exudate by rotifers. Further understanding of the role of Rotimer, which protects these micro-metazoans from neurotoxic aggregates (beta-amyloids, alpha-synucleins, or prions) [86], and the examination of its components may pave the way for promising research directions in the future, where the goal can be developing drug candidates against neurodegeneration.

4. Conclusion

In our previous studies with rotifers, a unique phenomenon was described, namely the exceptional ability of these animals to inactivate and utilize human neurotoxic aggregates such as beta-amyloid, alpha-synuclein, or prions as a source of nutrition through catabolism. The role and correlation of the rotifer-specific biopolymer (specifically Rotimer) with degradation processes were revealed during the investigation of the underlying mechanisms. After gaining knowledge about the physico-chemical and biological regulation of Rotimer, the main objective was to determine and predict the composition of this biomaterial to deepen our understanding of its natural role, functioning, and possible applications.

All exogenous samples derived from rotifers which are suspected to contain Rotimer elements were examined. The phenomenon that rotifers not only deposit their biopolymer on the surface of inductor particles, resulting in the RIC, but also initiate and facilitate humidity- and Ca^{2+}-dependent biofilm formation on the surface of the culture medium was discovered. Their full activity is exerted on the lower half of the formed layer.

By sequencing the genome and transcriptome of *E. dilatata* species and primarily by analyzing the protein content of the product of both

genders using MS, the putative main components of Rotimer were identified. The predicted, evolutionary conserved, and multifunctional rotifer-biopolymer forming proteins are the giant matricellular SSPOs, associated with adaptor 14–3-3 protein. In addition, there may be other uncharacterized proteins, such as elements bound to or trapped in the solidified exudate; however, the major hits seem certain to come from the protein family mentioned above. The anti-SSPO antibody-based antagonistic inhibition of the Rotimer secretion and, thereby the RIC formation process in monogonont rotifers may be a pivotal experimental result. Additionally, these results suggest that the SSPOs are main components of the Rotimer.

Considering the known properties of SSPOs and the Rotimer-related experimental results, theoretical parallelism and similarity can be drawn between the production of the external RIC network and the internal macromolecular processes in CSF taking place in the ontogenesis of vertebrates. The formation and molecular mechanism of RF complexes that primarily contain SSPO isoforms in the embryonal central nervous system may be an adequate analog process with producing the rotifer-specific exudate fibers.

The currently revealed results can be utilized at several points. The mechanism of the RIC formation can be a fast and adequate model system for the study of RF; moreover, the unique amino acid sequence motifs of these Rotimer proteins in this study hold promising potential as a starting point in interdisciplinary biomedicine and drug development, for instance against neurodegenerative diseases.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2023.127157.

Ethical approval

All procedures were conducted following the consensually 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 the University of Szeged (Szeged, Hungary) protocols under the supervision of the Hungarian Academy of Sciences. All applicable institutional, national, and international guidelines for the use of animals were followed. Furthermore, the experiments were performed on rotifers as micro-invertebrates; therefore, no ethical permission was needed according to the existing ethical regulations.

CRediT authorship contribution statement

Zsolt Datki: Investigation, Formal analysis, Writing- Original draft preparation, Visualization, Funding acquisition, Project administration. Zsuzsanna Darula: Investigation, Software, Data Curation, Methodology, Validation; Viktor Vedelek: Formal analysis, Software, Data Curation, Methodology, Visualization, Conceptualization; Eva Hunyadi-Gulyas: Investigation, Validation, Formal analysis, Software, Data Curation; Brian J. Dingmann: Conceptualization, Writing - Review & Editing; Balazs Vedelek: Validation, Formal analysis, Software, Data Curation, Visualization, Conceptualization; Janos Kalman: Conceptualization, Funding acquisition, Project administration; Peter Urban: Investigation, Validation, Formal analysis, Software, Data Curation; Attila Gyenesei: Funding acquisition, Project administration; Zita Galik-Olah: Writing - Review & Editing, Validation, Project administration; Bence Galik: Software, Data Curation, Resources, Funding acquisition. Rita Sinka: Resources, Investigation, Formal analysis, Project administration, Writing-Reviewing and Editing.

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

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

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