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Original Research

Does a Diet Rich in the Bacterium *Rhodopirellula rubra* Improve *Daphnia magna* Performance?

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Abstract

Background: In the wild various organisms contribute to daphnids diet. This study, intendeds to evaluate the potential of the concentration of *Rhodopirellula rubra* as a single or supplementary food source for *Daphnia magna*. **Methods**: Feeding assays were performed according to standard guidelines for chronic assays (21 days), and life-history parameters and several biomarkers (protein content, oxidative stress, energetic reserves and pigments) were measured. Five food regimens were conducted with 20 individual replicates (A - *R. subcapitata*; 0.2 - suspension of *R. rubra* at 0.2 arbitrary units (AU); 0.4 - suspension of *R. rubra* at 0.4 AU; 0.2+A - suspension of *R. rubra* at 0.2+alga; 0.2+A-suspension of *R. rubra* at 0.4 AU + alga). Additionally, the effects of three diets (A, 0.2, and 0.2+A) on the longevity of *D. magna* were assessed. **Results**: The five diets showed a different C, N, and carotenoids composition, with an increase in the mixed diets. The results confirmed that the mixed diets improved *D. magna* life-history parameters. A decrease in glycogen, and the increase of haemoglobin, protein, and gluthione-S-transferase (GST) were observed. Furthermore, *D. magna* fed with bacterial single diets, presented worsen life history parameters and a decrease in the protein content. An induction of oxidative stress response (increased catalase and GST), and a significant decrease in lipid peroxidation and an accumulation of glycogen and carotenoids were observed. Overall, an increase in the amount of *R. rubra* provided to *D. magna*, from 0.2 AU to 0.4 AU, negatively impacted daphnid performance. No significant effects on *Daphnia* longevity (a 110-day assay) were observed among the three diets tested. However, a significant survival percentage and fertility (cumulative offspring is more than twice) was observed when *D. magna* was fed with the mixed diet. **Conclusions**: Results demonstrated that different diets provided a nutritional diversified food to the daphnids that induced differences in *D. magna* performance. The mixed diets proved to be beneficial (with increase in offspring) on *D. magna* performance, independently of the bacterial concentration tested. When in single diet, bacterial concentration is not nutritionally sufficient to raise *D. magna* even when in increased concentration.

Keywords: life history; fecundity; carbon; nitrogen; proteins and carotenoids content; oxidative stress; haemoglobin; lipid peroxidation

1. Introduction

The freshwater crustacean species of the genus *Daphnia* is a versatile model organism that has long been used in several areas of research [1–7]. In addition, among the live zooplankton, *Daphnia* spp. are a valuable food for small freshwater fishes and are also used as an ingredient in the formulation of commercial foods [8–10]. Furthermore, *Daphnia* is recognized as a sentinel species of freshwater ecosystems because its decline is an indicator of environmental impacts [3]. This characteristic associated with parthenogenic reproduction, short life cycle, high fecundity, and easy laboratory maintenance, makes them commonly used tool in toxicity assessments [11–17]. Normally, these organisms are maintained in laboratory conditions during several generations for use in biological research. For the laboratory maintenance, one must consider several factors that influence the performance and the health of these organisms and the responses to experimental conditions tested. Diet, in terms of quality and quantity of food, is a predominant factor that impacts *Daphnia* performance due to its effects on life-history parameters such as growth, reproduction, and survival [18,19], and response in the presence of toxics [20–22]. The algae-based diet is standardized under laboratory conditions because of its sufficiency, reliability, and simplicity. However, reliance on a single carbon source can lead to fluctuations in *Daphnia* performance [23,24]. Indeed, previous studies have shown that Cladocera's exhibits different responses when subjected to different algae as food source [25,26]. Jonczyk *et al.* [27] reviewed notions about culture and maintenance in the laboratory and suggested that survival and reproduction in *Daphnia* were improved on mixed diets comparatively to a single algal diet. In fact, several studies addressed the effects of mixed diets on Cladocera's fitness [28–30], while oth-

ers evaluate alternative food sources that complement and diversify their diet [31,32].

Aquatic ecosystems have a considerable amount of bacteria which contribute to the diet of *Daphnia* spp. since they are non-selective filters. Previous studies conducted by Antunes *et al*. [33] and Marinho *et al*. [34,35] already showed the potential of Planctomycetes, namely *Rhodopirellula rubra*, to be a good supplementary food source when used in association with the standard food source (the microalgae *Raphidocelis subcapitata*), and its capacity to improve *Daphnia magna* life history parameters and in providing pink coloration. The authors also observed that these effects of *R. rubra* were more relevant at the exponential growth phase than the stationary growth phase. Furthermore, *D. magna* performance with mixed diets with two different planctomycetes, *R. rubra* and *Gemmata obscuriglobus*, was also assessed. Even though *G. obscuriglobus* produces sterols, molecules fundamental in nutritional terms for *D. magna*, *R. rubra* induced a better *D. magna* performance [34]. Also, an increase of MUFAs content in *D. magna* fatty acid' profile associated with the diet rich in *R. rubra* was found and the pink color displayed by bacteria was retained by mothers and observed in the offspring [35]. However, *R. rubra* showed not to be sufficient when used as a single food. In face of these results, one may question if the amount of bacterium provided as food has been sufficient for the welfare of *D. magna* as well as its ability to grow, reproduce and survive? Several studies reported that the amount of food affects growth, longevity, and reproduction in *Daphnia* spp. [18,19,36]. So, the question about bacterial concentration used in our previous studies for *D. magna* was raised. It is still unclear if the previous concentration of bacterium provided was sufficient regarding the nutritional value, and which are the physiological consequences of the increase of bacterial concentration on *D. magna*. In natural habitats, organisms are subject to wide seasonal variations in food concentration [37] in contrast to a constant food supply under laboratorial conditions. The responses in life history parameters of aquatic organisms may be related to variation in food availability being indicative of the status of the ecosystem.

As a follow up of our previous studies, we aimed to evaluate the adequacy of a single *R. rubra* diet and a mixed diet (*R. rubra* plus *R. subcapitata*) in two different bacterial concentrations in the *D. magna* performance along a chronic exposure (21 days). This assessment was based on the analysis of life-history parameters, and several physiological parameters: energetic reserves-protein and glycogen content; pigments-carotenoids and haemoglobin content; oxidative stress-antioxidant catalase (CAT) activity, detoxification glutathione S-transferase (GST) activity and lipid peroxidation (thiobarbituric acid assay-TBARS levels), and also on carbon, nitrogen, and carotenoids contents of the diets Furthermore, the effect of diets with the low amount of bacterium in *D. magna* longevity was also evaluated, regarding the life-history parameters.

2. Materials and Methods

2.1 Cultures of Organisms

The planctomycete *R. rubra* LF2 was isolated from the biofilm community of the marine macroalga *Laminaria* sp. from the north coast of Portugal [38]. *R. rubra* strain LF2 was first grown on solid modified M13 medium [38] at 26 °C and then transferred into liquid modified M13 medium with continuous stirring at 200 rpm. The culture was upscaled each three days, in exponential growth phase, starting with a culture volume of 50 mL, passed to 250 mL and finally to 1.5 L using always a 1:10 volume of inoculum. Cells in exponential growth phase (3 days of growth) were collected by centrifugation at 4000 rpm for 10 min. The cell pellets were resuspended in distilled water and the optical density adjusted at λ = 600 nm to 0.2 or 0.4 arbitrary units (AU). The cell suspensions were divided into aliquots of 50 mL and stored at –20 °C for later use in the feeding assays. Before being used in the feeding assays and due to the formation of cell clusters, the bacteria cell suspension was defrosted and subsequently sonicated for 1 to 2 min in a Misonix Microson Ultrasonic Cell Disruptor XL at 10 watts intensity. After this procedure, the cell suspension was again adjusted to 0.2 AU or 0.4 AU at λ = 600 nm, before being provided as food for *D. magna* in the feeding assays.

D. magna monoclonal cultures were maintained over several generations of pure parthenogenetic cultures under controlled conditions of temperature (20 ± 2 °C) and photoperiod (16 h^L:8 h^D). Cultures with 30 organisms were maintained in 500 mL of the synthetic medium, ASTM hard water according to standard procedures [39]. To provide essential elements to *Daphnia*, an organic additive (suspension extracted from brown algae *Ascophyllum nodosum*) [40] was added to the cultures. The culture medium was renewed every two days and *D. magna* was fed with the microalgae *R. subcapitata* with a ratio of 3.0 × 10^5 cells·mL^{-1}·day^{-1}. Neonates born between the 3rd and the 5th brood, with less than 24 h old, were used to establish a new culture or to initiate the feeding assays. The microalga *R. subcapitata* Korshikov (Hindak) (formerly known as *Selenastrum capricornutum* and *Pseudokirchneriella subcapitata*) was kept in cultures with Woods Hole MBL medium [41], under controlled conditions of temperature (20 ± 2 °C) and continuous light (~6000 lux). The microalga culture was cyclically renewed in the exponential growth phase (5–7 days old) and inoculated in fresh Woods Hole MBL medium [42,43]. Algal cell concentration was calculated based on the correlation of absorbance measured at λ = 440 nm and cell concentration previously determined [44].

Carbon, nitrogen, and carotenoids content were quantified in the five food regimens tested (Table 1). To quantify carbon in *R. rubra*, 2.5 mL of the sample at 0.2 AU and 0.4 AU, was centrifuged at 13,000 rpm for 60 sec-

Table 1. Composition of the diets provided in the feeding assay of *D. magna*, and diets characterization of carbon, nitrogen and carotenoids contents.

	A	0.2	0.4	0.2+A	0.2+A
R. subcapitata (cells·mL^{-1}·day^{-1})	3.0×10^5			3.0×10^5	3.0×10^5
R. rubra (AU·day^{-1})		2500 μL at 0.2	2500 μL at 0.4	2500 μL at 0.2	2500 μL at 0.4
Carbon (mgC·sample diet^{-1})	0.198	0.165	0.344	0.329	0.536
Nitrogen (mgC·sample diet^{-1})	0.0240	0.0423	0.0922	0.0688	0.118
Carotenoids (mg·L^{-1})	1.20 ± 0.10	0.038 ± 0.007	0.063 ± 0.012	1.68 ± 0.29	2.15 ± 0.34

onds. The supernatant was discarded, and the pellets were lyophilized for subsequent quantification For *R. subcapitata* 3×10^5 cells·mL^{-1} suspension was centrifuged at 13,000 rpm for 60 seconds. The supernatant was discarded, and the pellet was lyophilized for subsequent carbon and nitrogen quantifications. For C quantification each sample was carefully wrapped in tin foil, making sure there was no remaining atmospheric air inside. Before the analysis, blank experiments were run, and three measurements with D-phenylalanine (used as CHN standard) were made to obtain an average daily factor, to properly correct the measured results. The analyses were performed in a CHNS analyzer from Elementar® (model Vario MACRO Cube), equipped with a combustion tube set at 1050 °C, a reduction tube set at 850 °C and three adsorption columns, each one with a specific filling and set at a specific temperature to adsorb CO_2, H_2O and SO_2. These columns were then heated up sequentially to release the adsorbed gas to be detected by a Thermal Conductivity Detector (TCD). The gases were always detected in the following order: N_2 followed by CO_2, H_2O and finally SO_2. The analyses were performed and registered in VarioMACRO software version 4.0.8.

Carotenoids content was quantified by the method described by Zhang and Hu [45], where 10 mL of acetone (99.5%; V1) was added to the cell pellet from culture cell suspension (V2) and sonicated for 3 min in a Misonix Microson Ultrasonic Cell Disruptor XL, at 20 watts. Then, the tubes were kept in a water bath at 20 °C for 10 min. After centrifugation at 3000 rpm for 10 min, the total carotenoids were determined by reading the absorbance at 480 nm (A) and applying the following equation [45]:

$$\text{Carotenoids yield } \left(\text{mg} \cdot \text{L}^{-1}\right) = \text{AV1}/0.16\text{V2}$$

2.2 Feeding Assays

To evaluate the potential of *R. rubra* as a nutritional and supplementary food source for *D. magna*, feeding assays were performed according to standard protocols for assessing chronic toxicity [46,47]. The feeding assays had a duration of 21 days and were conducted under the same conditions as described for cultures maintenance. The experimental setup comprised 5 food regimens as describe in Table 1.

For each food regimen, twenty individual replicates were placed in a single glass vial filled with 50 mL of the synthetic ASTM hard water medium [39]. Daily, the daphniids were fed with corresponding food regimen (Table 1), and the medium renewed was conducted every two days. Mortality and the reproductive state were checked daily, and the neonates born during the assay were counted and discarded. At the end of the assay, the following endpoints were quantified: age at first reproduction (days), reproductive output (mean of offspring produced by all the mothers), fecundity (mean of offspring produced by the survivor mother at the end of the assay), number of broods, fecundity of the first brood, somatic growth rate (day^{-1}) and rate of population increase (*r*, day^{-1}).

Somatic growth rate was determined regarding the difference between the initial and final body size of the organisms, measured from the top of the head to the base of the caudal spine, in a binocular stereoscope. At the beginning of the assay, average of initial body length was calculated in a sub-sample of 20 neonates from the same brood of the organisms used in the assay. At the end of the feeding assay all the survivor organisms were measured. The somatic growth rate was calculated, according to the following expression:

$$\textit{Somatic growth rate} = \frac{(\text{Ln}(Lf) - (\text{Ln}(Li)}{\Delta t}$$

where L_f stands for body size (mm) of the organism at the end of the assay, L_i is the average body size (mm) of a subsample (n = 20) of neonates, and Δt is the duration of the assay (in days).

Survival and fecundity related data were used for the estimation of the per capita intrinsic rate of population increase (*r*), which was iterated from the Euler–Lotka equation:

$$1 = \sum_{x=0}^{n} e^{-rx} l_x m_x$$

where *r* is the intrinsic rate of increase (day^{-1}), *x* is the age class in days (0 … n), *lx* is the probability of surviving to age x, and *mx* is the fecundity at age x. Standard errors for *r* were estimated using the jack-knifing technique described by Meyer *et al.* [48].

At the end of the feeding assay, after body length and weight measurements, 3 daphnids were collected for

carotenoids quantification. Firstly, the organisms were placed in a vessel containing ASTM medium for 2 hours to promote gut cleaning, and afterward were storage in Eppendorf microtubes for carotenoids quantification. For each food regimen, the remaining organisms were storage in 5 individual groups at –20 °C for the next day quantifications (protein, glycogen, haemoglobin, carotenoid contents and the activity of catalase (CAT) and of glutathione S-transferase (GST), and levels of lipid peroxidation).

2.3 Longevity Assay

Regarding the results obtained in the feeding assay and to assess the potential effects of different food regimens on the longevity of *D. magna*, a new assay was performed with 20 individualized neonates exposed to the feeding regimens: A (standard food), 0.2 and 0.2+A (Table 1). The food regimens selected to assess the *D. magna* longevity were chosen according to the results obtained in the life-history parameters of the previous feeding assays. The assay was performed in vessels with 30 mL of ASTM medium and the organisms were kept under the same conditions as described for the culture's maintenance and the feeding assay. Culture medium were completely renewed three times a week and daphniids feed at the same time (fed three days a week, as described in OECD guidelines [47] — "the food provided to organisms should preferably be done daily, or at least three times a week when the medium is changed"). The organisms were checked daily for mortality, and if there were neonates, these were counted and discarded. The endpoints measured at the end of the assay were: survival, age at first reproduction (days), reproductive output, fecundity of the first brood, and rate of population increase (r, day^{-1}). The assay finished when the death of all organisms was observed at least in one of the feed treatments.

2.4 Biochemical Determinations

At the end of the feeding assay a set of biomarkers were measured in the organisms exposed to the different food regimens: energetic reserves-protein and glycogen content; pigments-carotenoid and haemoglobin content; oxidative stress-antioxidant catalase (CAT) activity, detoxification glutathione S-transferase (GST) activity and lipid peroxidation (thiobarbituric acid assay-TBARS levels). Organisms' homogenization was performed in 1.2 mL of cold phosphate buffer (50 mM, pH = 7.0 with 0.1% Triton X-100), and the homogenates were centrifuged at 13,400 rpm for 6 min at 4 °C. The supernatants were recovered for the biochemical analysis. The supernatant was divided into aliquots for subsequent determination of biochemical biomarkers, and all endpoints were determined in triplicate. The absorbances (except for haemoglobin determination) were performed in a microplate reader Thermo Scientific Multiskan GO spectrophotometer, version 1.00.40, with SkanIt Software 3.2.

Haemoglobin was determined by reading the absorbance of 1 mL of supernatant from each homogenized sample in a UV-1600 PC Spectrophotometer from 350 to 500 nm at 1 nm interval [49]. The specific absorbance of haemoglobin at 414 nm was normalized according to the technique described by Williams *et al.* [49]. The slope of the absorption spectrum within the wavelength intervals of 370–470 nm was determined by linear regression, from which the peak of haemoglobin specific absorbance (394–434 nm) was omitted. As for the 370–395 nm and 430–470 nm intervals, the corresponding values belong to non-haemoglobin materials that are present in *Daphnia* tissues. The observed absorbance value at the haemoglobin peak (414 nm) was subtracted by the expected absorbance value (calculated by linear regression calculated in the omitted interval of absorbance).

The glycogen quantification was conducted according to Lo *et al.* [50], where 100 μL of 30% KOH, saturated with Na_2SO_4, was added to 150 μL of homogenized supernatant. The suspension was boiled in a water bath (100 °C) for 10 min, and afterward placed on ice for 5 min. After cooling, 250 μL of ethanol (96%) were added and incubated for 10 min on ice (separation of glycogen from other saccharides-alkaline digestion). After this time, the sample was centrifuged at 1,550 rpm for 10 min at 4 °C and the pellet was resuspended in 125 μL of distilled water and homogenized. To each sample such as for glycogen standards (0, 3.125, 6.25, 12.5, 25, 50, and 100 μg·mL^{-1}), 125 μL of 5% phenol and 625 μL of H_2SO_4 were added and kept in ice for 10 min. The absorbance of the samples and the glycogen standards were read in quadruplicate in a spectrophotometer at λ = 490 nm. A standard curve was built regarding the values of the absorbance of glycogen standard concentrations and the results were calculated following the equation:

$$\mathrm{Y(abs)} = 0.00324\mathrm{x} + 0.07010$$

where Y is the absorbance values in the sample and x is the glycogen content (μg·mL^{-1}).

Catalase activity was determined following the procedure by Aebi [51], where the degradation of H_2O_2 to H_2O and O_2 decreases absorbance at a wavelength of 240 nm. The results were expressed by considering equivalent one unit of CAT activity to the number of moles of H_2O_2 consumed per minute per milligram of protein.

Glutathione S-transferase activity was measured according to the method by Habig *et al.* [52]. GST catalysis the conjugation of glutathione with the substrate 1-chloro-2,4-dinitrobenzene (CDNB), forming a thioether that increases absorbance at 340 nm. The results were expressed considering the equivalent one unit of GST activity to the number of moles of thioether produced per minute per milligram of protein.

Lipid peroxidation was measured by the quantification of the concentration of thiobarbituric acid reactive substances (TBARS), according to Buege and Aust

et al. [53]. The main by-products of oxidative damage to lipids membranes caused by reactive oxygen species (ROS) are malondialdehyde (MDA) and MDA-like compounds. This methodology is based on the reaction of compounds, such as MDA, formed by degradation of initial products from lipid membranes by free radical attack, with 2-thiobarbituric acid (TBA). Absorbance readings of each sample were measured at a wavelength of 535 nm and expressed as MDA equivalents per milligram of protein.

Protein was determined according to the methodology described by Bradford [54], adapted to microplates. This involves the binding of a dye (Bradford reagent) to the total protein, giving rise to a stable and colored complex that can be quantified at 595 nm. The γ-globulin (1 mg·mL^{-1}) was used as standard.

For the quantification of the carotenoid content 0.5 mL of 100% ethanol was added to the samples. The microtubes were wrapped in aluminum foil for protection from light and placed in the refrigerator overnight at –4 °C, according to Moeller *et al*. [55]. The day after, the total carotenoid absorption of the supernatant was measured in the spectrophotometer at λ = 450 nm. The extraction solution was used as blank. The wavelength of 450 nm is very close to the maximum absorption of β-carotene; therefore, the total carotenoid content was quantified according to the following equation Moeller *et al*. [55]:

$$\text{Total carotenoids } \left(\mu g \cdot mg^{-1}\right) = 1 \times 10^4 x \left(OD_{450}/2620\right) \times (V/W)$$

where V is the extract volume (mL), W is total dry weight (mg) of organisms and OD = optical density (λ).

2.5 Statistical Analysis

One-way Analysis of Variance (ANOVA) was applied at all the endpoints measured in the feeding and longevity assays: age at first reproduction, fecundity, reproductive output, number of broods, number of N1 offspring, somatic growth rate and rate of population increase (these endpoints were log (x + 1) transformed prior to the ANOVA, to comply with ANOVA requirements), glycogen, protein, TBARS, GST and CAT. When ANOVA results showed significant differences a post-hoc Tukey test was conducted to assess statistical differences between the different food regimens. For all analyses, the level of significance (α) used was 0.05.

3. Results and Discussion

3.1 Feeding Assays, Life-History Parameters

One of the main questions addressed in this work was if an increase in the amount of *R. rubra* provided to *D. magna* as food would enhance its growth. As previously observed by Marinho *et al*. [34,35], *R. rubra* at a concentration of 0.2 AU proved not to be sufficient to provide the nutritional requirements needed by *D. magna*. In fact, a significant delay of the age at first reproduction, a decrease of broods, and an increase of somatic growth rate, fecundity, reproductive output, and the rate of population increase were observed (Fig. 1). The obtained results suggest that 0.2 *R. rubra* concentration, when provided as exclusively food source, is below the "incipient limiting level" (ILL—the external level above which there is no limiting effect of food supply, terminology described by Fry *et al*. [56], when the daphnids filter at a maximum rate [1,57]. Regarding the double *R. rubra* concentration (0.4), *D. magna* performance was not improved, and the parameters of the life history even aggravated (Fig. 1). The cell aggregate formation typical of *R. rubra* may justify this result, as the feeding structures of *D. magna* may be inadequate to efficiently filter the bacteria. In fact, and as already demonstrated by Gliwicz *et al*. [58], filtration rate and particle size affect the amount of food collected and ingested by *Daphnia* as well as in other organisms (e.g., rotifers [59]). On the other hand, the higher amount of food in diet 0.4 may reduce the ability of *Daphnia* to ingest the bacteria due to occlusion of the filter apparatus, fact already observed by Martínez-Jerónimo *et al*. [60]. Both food regimens (0.2 and 0.4) presented to be poor diets compared to the mixed diets (0.2+A and 0.2+A) and the diet with only alga (A).

On the other hand, when *D. magna* was fed with the mixed diet 0.2+A, a significant reduction of the age at first reproduction, and a significant increase of somatic growth rate, fecundity, reproductive output and rate of population increase were recorded (Fig. 1), corroborating the data from Marinho *et al*. [34,35]. Moreover, when comparing the two mixed diets (0.2+A and 0.2+A), no differences were observed (a similar *D. magna* behavior was obtained; Fig. 1). This implies that, under the same laboratorial conditions, *D. magna* did not need an additional quantity of bacteria to achieve the same levels of reproduction and growth. The elemental analyses of the levels of C and N in the five diets showed an increase from the single to the mixed diets (Table 1). This carbon increase in the diets, from A to 0.4 or from 0.2+A to 0.2+A, showed to be insufficient to improve *D. magna* life-history parameters. On the other hand, the quality of the food provided by the alga (although comparatively lower) is essential to *D. magna* rearing. Curiously, the levels of N present in the bacterium are higher in all diets than in the algal diet. The level of this element may explain the important role of this bacterium in the N availability to *D. magna* in the mixed diets. Taipale *et al*. [28] and Freese *et al*. [29], also recorded that when bacteria [*Micrococcus luteus*, *Methylomonas methanica*, *Methylosinus trichosporium* (2.5 mg·C·L^{-1} of bacteria in 5 mg·C·L^{-1} of total food concentration), *Escherichia coli* (4.4 × 10^6 cell·mL^{-1}) and *Flavobacterium* sp. (4 × 10^6 cell·mL^{-1})] were used as supplement to algae diets, an increase of somatic growth rate and reproduction of *Daphnia* was observed, when compared to an exclusive algal diet. Indeed, two different carbon sources provided in the diet (from bacteria and from alga—Table 1) improved the nutritional di-

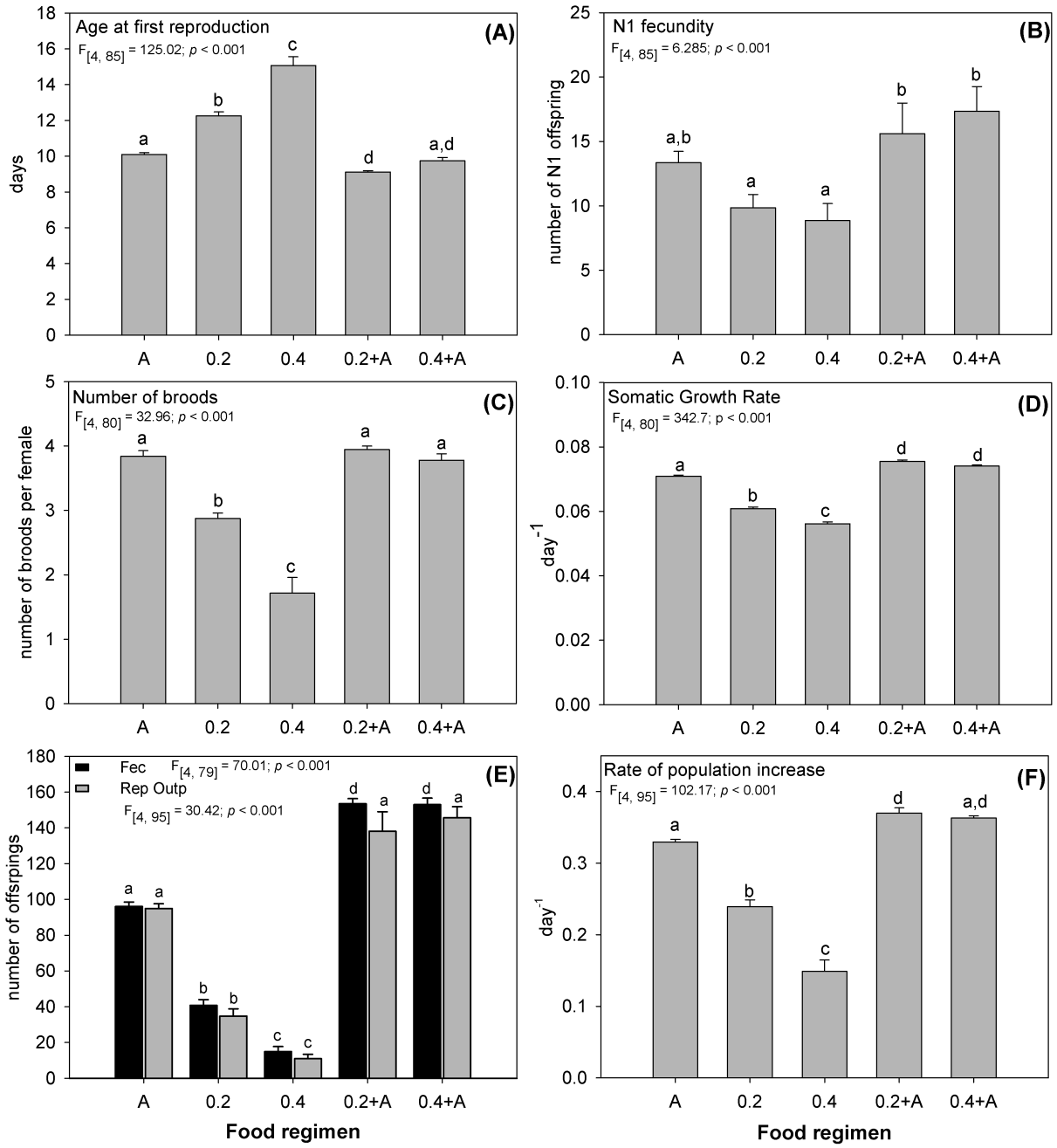

Fig. 1. Life-history parameters; (A) age at first reproduction; (B) offspring of first brood; (C) number of broods; (D) somatic growth rate; (E) fecundity and reproductive output; and (F) rate of population increase, results of *D. magna* after exposure for 21 days to several food regimens (see Table 1). Error bars represent standard error (n = 20) and different letters (a,b,c,d) represent significant differences between food regimens (Tukey test, $p < 0.05$).

versity of the diet, which implies a greater availability of carbon for the growth of primary consumers such as *Daphnia* [61].

3.2 Feeding Assays, Biochemical Parameters

The effects of the different diets on *D. magna* physiological parameters were also evaluated and are presented in Figs. 2,3. The protein levels recorded in 0.2 and 0.2+A were similar to the A diet, while in 0.4 and 0.2+A a significant decrease (0.7 ×) and increase (1.6 ×), respectively, were observed (Fig. 2A). The nutritional value of a diet is determined by the level of essential elements, such as nitrogen, phosphorus, and carbon, which are important for zooplankton growth and reproduction and for the competitive abilities of various zooplankton species [62,63]. Furthermore, proteins, lipids and carbohydrates are also important since these nutrients are essential molecules and reserves necessary for the enhancement and the effectiveness of growth

and reproduction [35,64,65]. Although daphnids can accumulate large amounts of proteins, this content can be influenced by the age of organisms, the diet provided and the number of eggs produced [66,67]. Protein content reflects the entire physiological state of the organism and a measure of its energy balance [68]. Our results showed the highest protein content in organisms fed with the mixed diet 0.2+A, demonstrating that this diet is the more suitable for protein production and that 0.4 diet is the less adequate to fed *D. magna*. Indeed, the diets characterization (Table 1) showed an increase of C, N, and carotenoids content in the mixed diets, demonstrated higher diversity and nutritional food provided to *D. magna* with essential elements to the *Daphnia* development (Fig. 1).

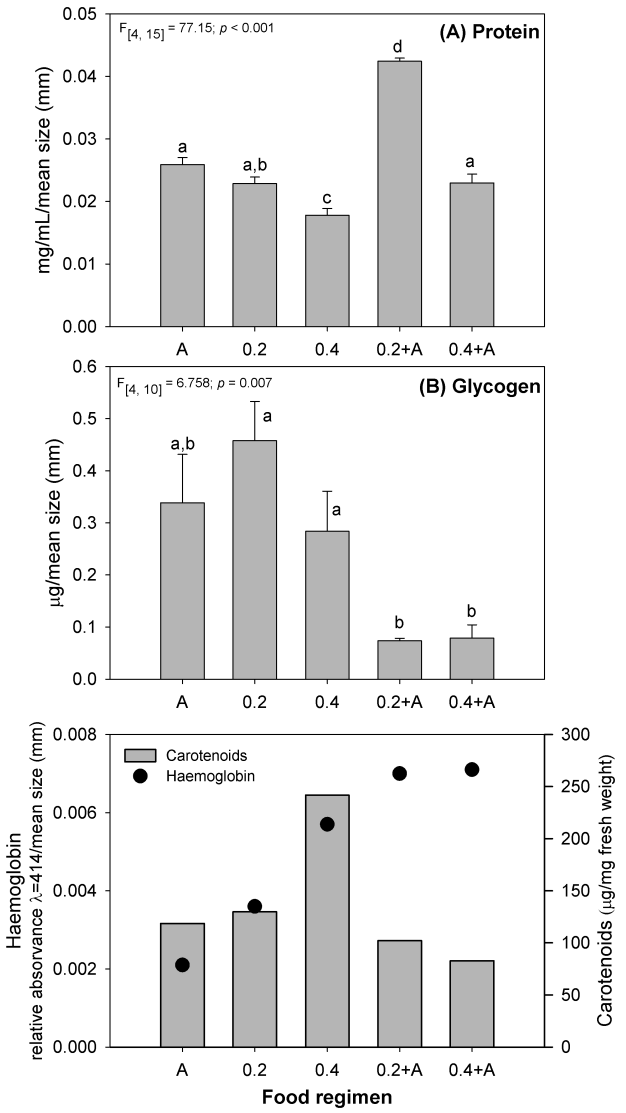

Fig. 2. Variation in protein (A) glycogen, (B) haemoglobin and carotenoids in *D. magna* after exposure for 21 days to several feeding regimes (see Table 1). Error bars represent standard error (n = 20), different letters (a,b,c,d) represent significant differences between food levels (Tukey test, $p < 0.05$).

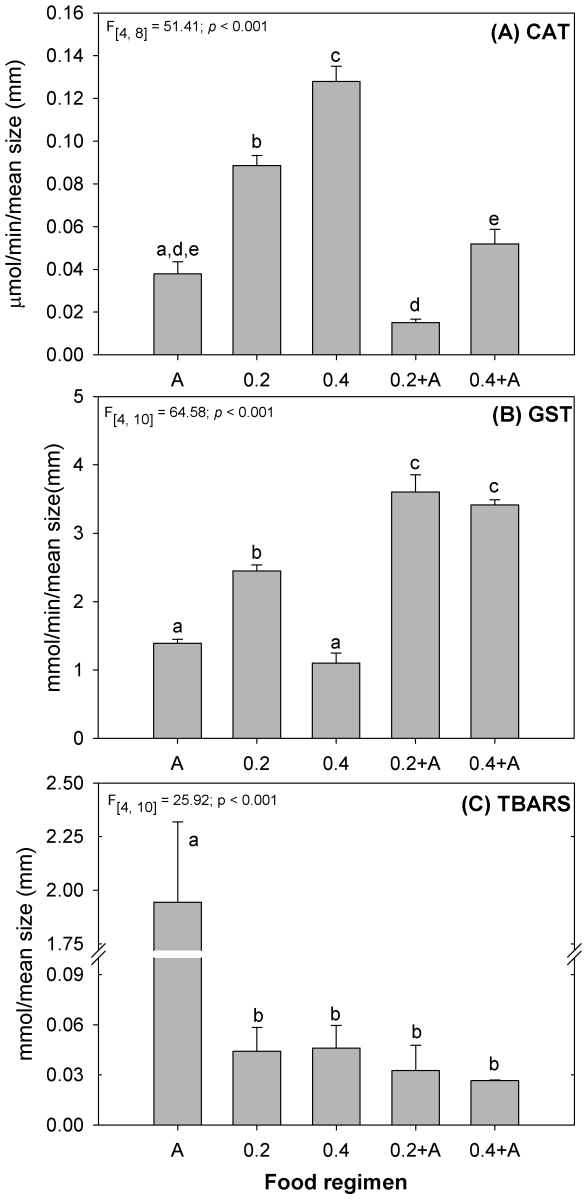

Fig. 3. Results of oxidative stress biomarkers (A) CAT; and (B) GST activities; (C) TBARS concentrations, in *D. magna* after exposure for 21 days to several feeding regimens (see Table 1). Error bars represent standard error (n = 20) and different letters (a,b,c,d,e) represent significant differences between food levels (Tukey test, $p < 0.05$).

Glycogen represents the main form of glucose storage in animal organisms, which fuels glycolysis as a first response in the case of a lack of food [69,70]. In the 0.2 and 0.4 diets, *D. magna* had no difference in the glycogen levels comparatively to A diet (Fig. 2B). Indeed, a significant lower somatic growth rate and reproduction values (see Fig. 1D–F) were observed in the single bacterial diets, showing that the organisms allocated the energy available for self-maintenance instead of supporting growth and reproduction. Several studies already demonstrated that un-

der conditions of low feeding, daphnids increase the allocation of energy for self-maintenance [71,72]. Regarding the glycogen contents in the mixed diets, a significant decrease was observed relatively to the bacterial single diets (Fig. 2B). As a significant increase of somatic growth rate and reproductive parameters was observed in the mixed diets (Fig. 1D, E), *D. magna* was not able to accumulate glycogen due to consumption for reproductive needs. This indicates that *D. magna* energy allocation strategy was directed for growth and reproduction having not the possibility for reserve storage. When glycogen is used for energy production it is rapidly catabolized, leading to large losses of this energy reserve [68].

Haemoglobin in *Daphnia* is a respiratory pigment, which is part of the oxygen transport system [73]. In invertebrates, haemoglobin is involved in adaptive response to changes in environmental conditions, such as oxygen availability, pH, salinity, CO_2, sulfides, carbon monoxide and temperature [74]. Haemoglobin as to be synthetized by *Daphnia* with intake of matter and energy [75]. Fox [76] verified that *Daphnia* does not synthetize this pigment under insufficient food conditions as well-fed organisms do. Our results showed that haemoglobin levels increased in all diets provided, with the highest values recorded for the two mixed diets (Fig. 2C). Even though the increase in haemoglobin concentration recorded in the mixed diets (Fig. 2), no effects were observed in the life-history parameters on *D. magna* (Fig. 1). Schwerin *et al*. [77] reported that, under laboratory conditions with ideal feeding regimens, *D. galeata-hya*lina showed higher haemoglobin concentrations.

Several different types of carotenoids are present in algae and bacteria. *R. subcapitata* belongs to the phylum Chlorophyta which contains, among others, β-carotene, violaxanthin, *9'-cis* neoxanthine and lutein, as well as chlorophyll a and b [78]. The Planctomycetota *R. rubra* possesses three saproxanthin-type carotenoids, including a rare C_{45} carotenoid [79]. Animals do not synthesize carotenoids *de novo* and they obtain them from food or through modified metabolic reactions [80]. In addition, carotenoids are important for immunity, non-enzymatic antioxidant defense, photoprotection against photodegradation and contribute to the increase of reproduction [80]. The quantification of carotenoids in the different food regimens is shown in Table 1 and Fig. 2 shows the carotenoids content for *D. magna* feed with the different food regimens. Our results showed that the levels of carotenoids quantified in *D. magna* were highly increased in the diet 0.4 (2.0 $\times$) while in the mixed diets a decrease ($\approx$ 0.7 $\times$) was observed (Fig. 2C). Therefore, these last results are somehow unexpected due to the high levels of pigments provided by the two mixed diets, specially the 0.2+A (Table 1). This suggests that the highest levels of carotenoids provided by the mixed diets (Table 1) were used for reproductive purposes of the daphnids, evidenced by the increase of the reproductive output (Fig. 1E). Similarly, Schneider *et al*. [81] also observed a depletion of carotenoids in the copepod *Leptodiaptomus minutus* during the increase of offspring production. On the other hand, the two bacterial diets showed low and similar levels of carotenoids (Table 1). As these diets showed a significant decrease in the reproductive output, higher levels of these pigments were observed in *D. magna* fed with the two single bacterium diets (Fig. 1E). Comparing in between the two single diets, carotenoids levels in the diet 0.2 were lower than in diet 0.4, which may be due to pigment transfer to a greater number of neonates. This suggests that the mothers in the 0.4 diet accumulated the pigment, as there was a decrease in fertility despite the increased concentration of the bacterium. Hairston Jr [82] collected, at regular intervals, zooplankton samples from two central Washington lakes and observed that adult females of two copepod *Diaptomus* species (*Diaptomus sicilis* and *Diaptomus neuadensis*) accumulated carotenoids and transferred them to the eggs and nauplii. Indeed, Marinho *et al*. [35] already demonstrated that *D. magna* can absorb *R. rubra* carotenoids and transferred them to the next generation.

It is known that food deprivation or lack of nutritional factors, such as vitamins, causes changes in the activities of tissue antioxidant enzymes [83]. The effect of diet on free radicals' formation varies in function of the type of organism, age, physiological status, and ingested food [84]. For example, a study of fed and fasted mammalian species (male Wistar rats) showed that malnutrition accelerated the production of free radicals with the consequent depletion of the liver antioxidant stores [85]. If vitamins C and E were added to mice erythrocytes previously incubated with hydrogen peroxide (H_2O_2), a decrease in the plasma concentration of malondialdehyde, a product of lipid peroxidation, was observed [86]. Sharma *et al*. [87] observed an improvement in the antioxidant defense system (lower lipid peroxidation and higher activity of antioxidant enzymes (glutathione [GSH], GST, CAT and superoxide dismutase [SOD]) in muscle tissue of juvenile fish *Clarias gariepinu* fed with the microalgae *Ascochloris* spp. comparatively to organisms fed with a commercial diet. CAT is a protective enzyme responsible for the degradation of hydrogen peroxide and is present in almost all animal cells [88]. GSTs are present in all aerobic organisms, belong to the family of multifunctional cytosolic enzymes and are detoxifying enzymes [89]. Lipid peroxidation occurs when free radicals react with lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs) [90]. As noted by Harris [91], organisms always maintain basal levels of antioxidant enzymes activity. As it is known, oxygen is a highly reactive molecule, which can form free radicals or reactive oxygen species (ROS). When this happens, the cell's antioxidant systems kick in, and regulate the balance between ROS production. If this balance is disrupted, oxidative stress results, with alterations in cellular functions leading to various pathological conditions [88].

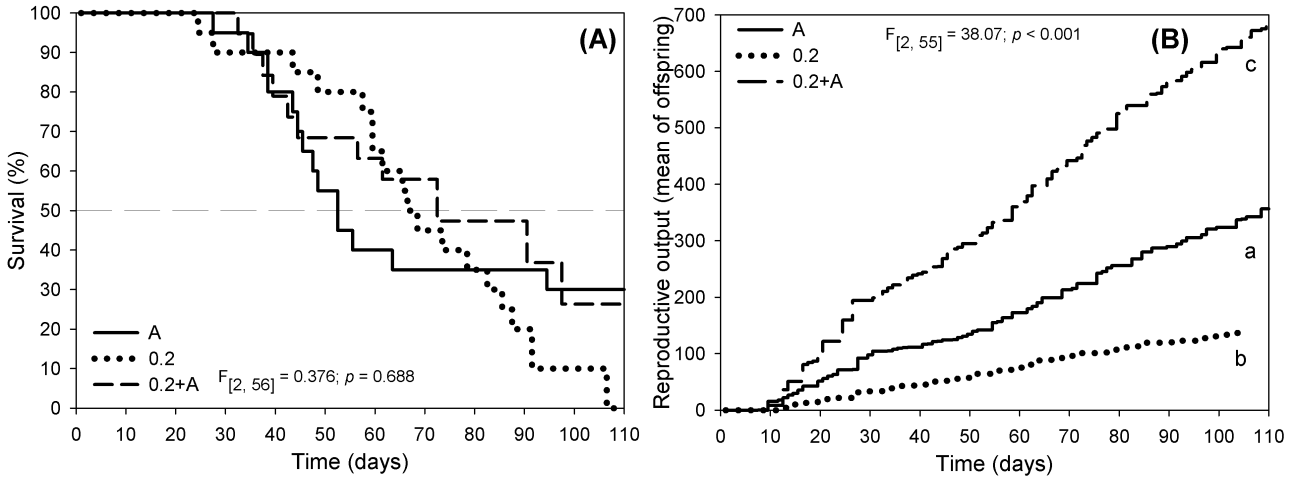

Fig. 4. Survival curves (A) and reproductive output (B) for *D. magna* fed with the green microalgae *R. subcapitata* (A), the planctomycete *R. rubra* suspension at 0.2 AU (0.2) and a mixed diet of *R. rubra* suspension at 0.2 AU plus *R. subcapitata* (0.2+A). Different letters (a,b,c) stands for significant differences between the food regimens (Tukey test, $p \leq 0.05$).

Results from the evaluation of oxidative stress in *D. magna* under the different feeding regimens are provided in Fig. 3. The levels of CAT activity in the mixed diets were similar to A diet, showing a non-oxidative stress response. CAT activity was significantly increased in *D. magna* fed with the two bacterial diets (Fig. 3A). This result may be justified by a potential increase in hydrogen peroxide levels and consequent oxidative stress due to *D. magna* undernutrition status (Fig. 3A —0.2 and 0.4). Im *et al.* [92] also demonstrated that under low food concentration and high temperatures *D. magna* showed increased activity of antioxidant enzymes (SOD and CAT) and reduced adult somatic growth rate. Regarding the GST activity, a significant increase in *D. magna* fed with the diets 0.2 (1.8 ×), 0.2+A (2.6 ×) and 0.2+A (2.5 ×) was observed (Fig. 3B). Under 0.2 diet the increase in GST values may be due to insufficient levels of food. On the other hand, Metcalfe *et al.* [93] described that reproduction increases oxidative stress and that this stress may tend to increase with the effort in the quantity and quality of offspring. This may explain the increase of GSTs values due to the high reproductive output recorded (Fig. 1E) in the mixed diets. A significant reduction of the lipid peroxidation was recorded in the bacterial diets and the mixed ones (Fig. 3C). The low levels observed in the four diets may be due to the high activities values of the antioxidant enzymes CAT and GST (Fig. 3A,B) and consequent improvement of *D. magna* antioxidant defense system [87]. In the algal diet A, the levels of peroxidation are within the values already observed for *D. magna* by Rodrigues *et al.* [17]. In fact, in this study a slightly higher values of lipid peroxidation were observed in the A diet (≈ 3.8 mmol·mg prot^{-1}), than the ones recorded in our study (2.1 mmol·mg prot^{-1}). Several studies also described that, between other functions, the GST prevents lipid peroxidation by reducing lipid hydroperoxides [94,95]. Among other functions, the antioxidant capacity of carotenoids, such as singlet oxygen ($^1O_2$) suppression, protects cells against free radicals and inhibits lipid peroxidation [96]. Furthermore, *R. rubra* possesses the carotenoid saproxanthin [79] that was already referred as leading to the reinforcement and stabilization of biological membranes and enhancing protection against radical-induced peroxidation [97]. The existence of this kind of carotenoids suggests that they may have been used by *D. magna* for detoxification, with a significant decrease in TBARS.

3.3 Longevity Assay

A longevity study (from birth to natural death) was also performed with *D. magna* fed with 3 feeding regimens (A, 0.2 and 0.2+A; Figs. 4,5). No death occurred in the first 25 days of the assay for any of the feeding regimens (Fig. 4A). After this period, survival felt abruptly from day 30 to day 60 in the A diet (with a slope of –1.039) with 50% death recorded at day 50 (Fig. 4A). In the 0.2 feeding regimen, death occurred from day 40 to day 90 (with a slope of –1.243) with 50% mortality around day 70, with complete organisms' death recorded at day 107 (Fig. 4A). In the 0.2+A feeding regimen, the decrease survival was from day 30 to day 100 (with a slope of –0.831) with 50% death occurring at day 70. No significant differences were recorded in *D. magna* survival (days) regarding the three feeding regimens tested (Fig. 4A). The reproductive output along the 110 days of experiment is shown in Fig. 4B. A significant decrease of reproductive output was recorded for the 0.2 diet (with a slope of 1.479), while a significant increase was observed for 0.2+A diet (with a much higher slope of 6.671) and for A diet (a slope of 3.405) (Fig. 4B). Although this assay was performed for 110 days instead of 21 days, comparable results were obtained (for comparison see Fig. 1). A significant delay in the age at first reproduc-

tion (Fig. 5A), and a significant decrease of N1 fecundity (Fig. 5B) were observed in 0.2 feeding regimen. The rate of population increase showed similar results obtained in the 21-day assay (Fig. 1F) where a significant decrease was observed for 0.2 while for 0.2+A a significant increase was recorded (Fig. 5C).

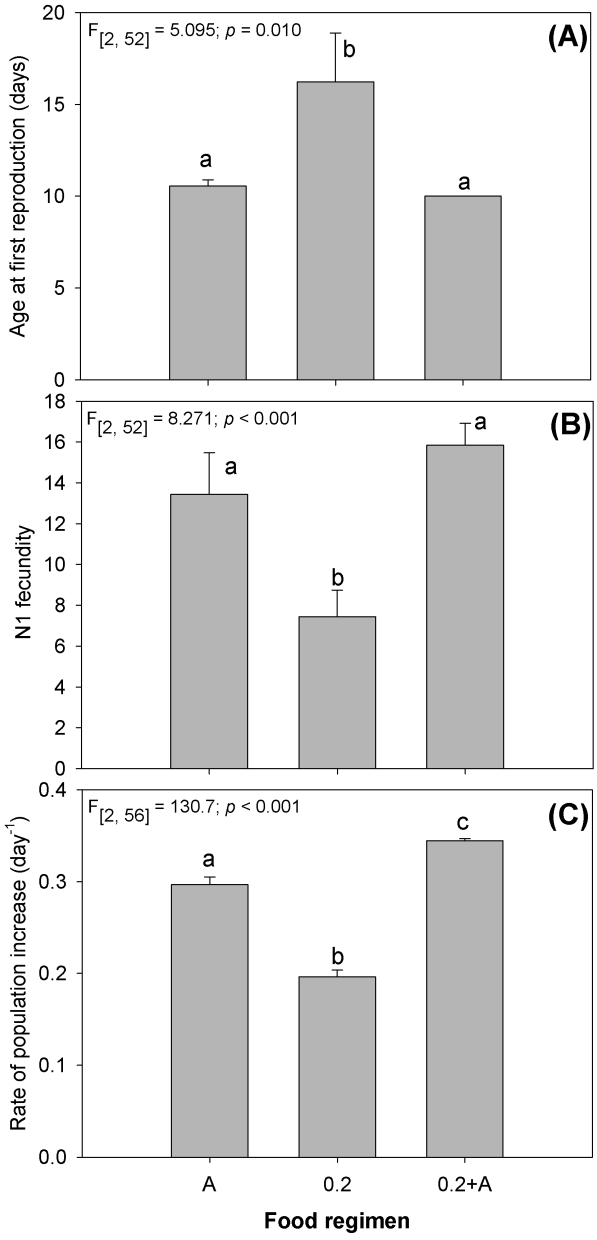

Fig. 5. Life history parameters (A) age at first reproduction; (B) offspring of first brood; and (C) rate of population increase in longevity assay of *D. magna* fed on the green microalgae *R. subcapitata* (A), the planctomycete *R. rubra* suspension at 0.2 AU (0.2) and a mixed diet of *R. rubra* suspension at 0.2 AU plus *R. subcapitata* (0.2+A). Error bars represent standard error (n = 20), and letters (a,b,c) stands significant differences between the food regimens (Tukey test, $p \leq 0.05$).

Vijverberg [98] observed that well fed *D. hyalina* lived for 43.4 days while poorly fed organisms lived for 64.4 days. Ingle [99] reported that well fed *D. longispina* organisms had an average life duration time of 29.9 days while malnourished organisms lived longer, for 41.4 days. Indeed, previous studies with *D. magna* also reported that survival increased under limiting food conditions [60]. *D. magna* fed with 0.05, 0.15, 0.5, 1.5, and 4.5 mgC·L^{-1} of *Scenedesmus obliquus* lived longer at the lowest C concentration (0.15 mgC·L^{-1}, 114 days) than at the highest concentration (4.5 mgC·L^{-1}, 65 days) [18]. The here-obtained results are not in agreement with these results because survival did not increase in the lower food condition. Pietrzak *et al.* [18] also observed that when organisms live longer, the fecundity was significantly reduced. In our study, the bacteria feeding regimen 0.2 induced a decrease of *D. magna* rate of population increase, due to investment of the energy in maintenance and survival at the expense of reproduction. This was already showed by a study performed by Antunes *et al.* [100] where different *Daphnia longispina* clones under starvation conditions for 21 days, registered a significant decreased in the reproductive output. Martínez-Jerónimo *et al.* [60] observed similar results in *D. magna* fed with low food concentration of the microalgae *Ankistrodesmus falcatus* and *Scenedesmus incrassatulus*, while, in the highest food densities a shorter life span, higher fecundity, and larger clutch sizes were observed. The here-obtained results also showed that the diet provided to *D. magna* influences longevity and reproduction performance. When the 0.2+A diet was supplied, individuals had similar longevity comparatively to the A diet when considering the 110 days, but obtained higher reproductive output, rate of population increase, and the offspring increased significantly. Although several studies demonstrated that longevity increases with lower food concentration [58,98,99], our results showed that the quality of food also affected the longevity. Furthermore, they also showed that in the feeding regimen with the highest diverse food sources (0.2+A), a higher *Daphnia* performance was recorded compared to the single diets (A and 0.2).

4. Conclusions

The elemental analyses of the levels of C, N, and carotenoids in the five diets showed overall an increase from single diets to the mixed diets. These results demonstrated that different diets (algae and bacterium) provided a diversified and different nutritional food to the daphnids that represent differences in the performance of *D. magna*, even in laboratory conditions.

The bacterium (*R. rubra*) proved to be a good supplement food for growing *D. magna* since the mixed feeding regimens 0.2+A and 0.2+A (bacteria + algae) significantly improved its performance, reinforcing the previous results obtained by our group. We also showed that a food regimen with higher *R. rubra* levels (from 0.2 to 0.4) is not enough to

fill *D. magna* nutritional needs. Moreover, these results are supported by the longevity assay. The high protein content observed showed that the mixed diet tested (0.2+A diet) is, in fact, the best diet for *D. magna*. Low levels of lipid peroxidation were registered in all bacterial diets which may be due to the levels of activity of the antioxidant enzymes (CAT and GST) observed, and the intake of carotenoids (non-enzymatic antioxidant defense) provided by the bacterium. Thus, our results evidenced that biological, biochemical, and physiological processes are affected by the food conditions to which organisms are subjected.

Thus, and reinforcing our previous studies, we can conclude that *R. rubra* is a good diet supplement for *D. magna* that improve the growth, fecundity and survival of *D. magna* under laboratorial conditions enhancing this daphnid' performance. Although different food sources are already used in the maintenance of *Daphnia* spp, a diversified diet, that includes the bacterium *R. rubra*, can be adopted to improve *D. magna* performance in laboratory maintenance.

Author Contributions

MM, GJ, OML and SCA contributed to the study conception and design. MM, GJ, JC, LS and SCA performed all material preparation, data collection and analysis. MM and GJ have written the first draft of the manuscript and all authors commented on the various versions of the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Conflict of Interest

The authors declare no conflict of interest.

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