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Phytohormone gibberellins treatment enhances multiple antibiotics removal efficiency of different bacteria-microalgae-fungi symbionts

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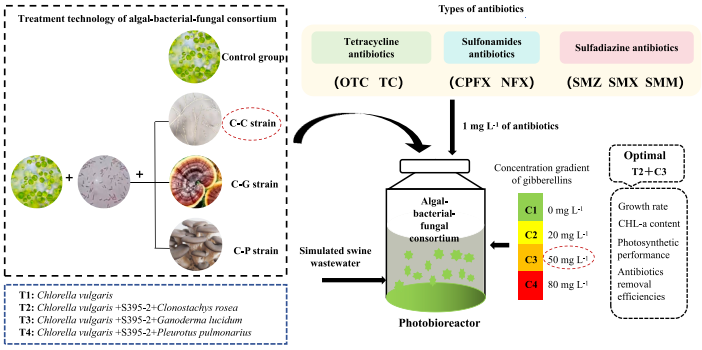
HIGHLIGHTS

- *C. vulgaris* -S395-2- *C. rosea* presents superior antibiotics removal capacities.
- The maximum TCs removal efficiencies were 97.0 ± 2.8 %.
- The induction of GAs enhanced the antibiotics purification of the algal system.
- The optimal GAs concentration was 50 mg/L.

GRAPHICAL ABSTRACT



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ABSTRACT

To develop and characterize novel antibiotics removal biomaterial technology, we constructed three different bacteria-microalgae-fungi consortiums containing *Chlorella vulgaris* (*C. vulgaris*), endophytic bacterium, *Clonostachys rosea* (*C. rosea*), *Ganoderma lucidum*, and *Pleurotus pulmonarius*. The results showed that under treatment with 50 mg/L of gibberellins (GAs), the three bacteria-microalgae-fungi symbionts had maximal growth rates (0.317 ± 0.030 d^{-1}) and the highest removal efficiency for seven different antibiotics. Among them, *C. vulgaris*-endophytic bacterium-*C. rosea* symbiont had the best performance, with antibiotics removal efficiencies of 96.0 ± 1.4 %, 91.1 ± 7.9 %, 48.7 ± 5.1 %, 34.6 ± 2.9 %, 61.0 ± 5.5 %, 63.7 ± 5.6 %, and 54.3 ± 4.9 % for tetracycline hydrochloride, oxytetracycline hydrochloride, ciprofloxacin, norfloxacin, sulfadiazine, sulfamethazine, and sulfamethoxazole, respectively. Overall, the present study demonstrates that 50 mg/L GAs enhances biomass production and antibiotics removal efficiency of bacteria-microalgae-fungi symbionts, providing a framework for future antibiotics-containing wastewater treatment using three-phase symbionts.

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1. Introduction

The swine industry benefits much from the use of large amounts of antibiotics but produces large-scale wastewater that is an important source of antibiotics in the environment, which might inhibit bacterial growth (Cheng et al., 2020). Environmental antibiotics may also unintentionally select antibiotic-resistant mutants and genes that might enter the water and food chain, causing a threat to human health. Tetracyclines (TCs), quinolones and sulfonamides are the characteristic antibiotics that are most extensively and frequently used in the swine industry (Cheng et al., 2020; Vaishnav et al., 2023). The scientific treatment of antibiotic contamination within piggery wastewater is still under academic and industrial investigation. The concentration of major antibiotics in swine wastewater has been measured using liquid chromatography (LC), high-performance liquid chromatography (HPLC) and gas chromatography techniques. The representative concentration values of these antibiotics include: Sulfonamides [$(5.03 \times 10^{-3} - 0.95) - (8.59 \times 10^{-3} - 1.59)$], Sulfamethazine (SMX, 0.44–324.40), Sulfamethoxazole (SMM, 14.05–316.50), Sulfadiazine (SMZ, 98.90), Sulfamonomethoxine (45.40), Tetracycline (TC, 1.45–388.70), Oxytetracycline (OTC, 5.33–25.36), Chlortetracycline (2.65–32.67), and Doxycycline (685.60) (Cheng et al., 2018).

Antibiotics have very low Henry's law constant values (k_H values ≈ 4.97E10^{-31}–1.58E10^{-10} mol m^{-3} Pa^{-1}) and are hardly removed by volatilization processing (Langbehn et al., 2021). Moreover, their photodegradation is usually restricted by very limited sunlight penetration (Tiwari et al., 2017). Currently, approaches used to remove antibiotics from wastewater are mainly physical and chemical methods and a combination of physical and chemical methods with biological ones (Hu et al., 2020). Physical approaches like adsorption usually remove antibiotic components in wastewater by adsorbing onto a porous solid material surface (Avcı et al., 2020). Chemical approaches, including chemical flocculation, precipitation, and advanced oxidation, can achieve a higher reaction rate and removal efficiency. Conversely, biological methods include aerobic digestion, artificial wetlands, traditional activated sludge treatment, and anaerobic digestion (Liu et al., 2023; Tang et al., 2023). Traditional activated sludge treatment mainly depends on sludge adsorption and rarely on the microbial degradation of antibiotics (Peng et al., 2019). Anaerobic digestion (biogas fermentation) is often applied to treat the nutrient-rich swine wastewater to degrade excess harmful substances such as antibiotics and produce methane (Deng et al., 2023). However, an economical, simple and effective method for treating antibiotics in wastewater is still necessary, and much effort is needed to achieve this goal worldwide.

Microalgae-based swine wastewater treatment technologies have attracted more attention (López-Sánchez et al., 2022) since symbionts composed of endophytic bacteria, microalgae and fungi have unique advantages in stress adaptation and antibiotic removal capability in more complex environments (Wang et al., 2023; Xu et al., 2021; Zhang et al., 2021a; Zhang et al., 2021b). Microalgae cells can be cultured in autotrophic, heterotrophic, or mixotrophic modes, enabling them to quickly adapt to complex and different culture conditions, such as swine wastewater, rich in organic and inorganic matter, e.g., nitrogen and phosphorous. The Microalgae symbiosis approach exhibits excellent application potential because of its relatively efficient treatment rate, cost-effectiveness and system stability. Microalgae or bacteria-microalgae-fungi symbioses have been reported to efficiently decrease antibiotic contamination in livestock wastewater (Ferrando & Matamoros, 2020). Among the diverse microalgae, *Chlorella vulgaris* (*C. vulgaris*) has relatively high growth and adaption capabilities to stressful conditions and enhanced antibiotic resistance, with the capacity to remove antibiotics like TC and sulfonamide (Rossi et al., 2020). The efficiency of microalgae monoculture-based removal of antibiotics from wastewater is usually low if the strain cells are in the growth stagnation stage. As for the bacteria-microalgae-fungi consortium, the algal symbionts more efficiently remove antibiotics from wastewater via various pathways, including adsorption, accumulation, and biodegradation (Leng et al., 2020). Algal symbionts can achieve enhanced nitrogen removal efficiency and performance compared with microalgal monoculture (Arun et al., 2019). Moreover, due to the complex composition of livestock wastewater, microalgal monoculture may be easily affected by different factors. Thus, the bacteria-microalgae-fungi consortium is recommended for purifying wastewater with complex composition (da Silva Rodrigues et al., 2020). The bacteria-microalgae-fungi symbionts also exhibit enhanced resistance to stressful conditions and are more cost-effective than microalgal monoculture (Wei et al., 2023).

Although the mechanisms by which microalgae remove antibiotics are still elusive, there are a few postulations: (1) microalgae absorb target antibiotics onto the cell surfaces; (2) antibiotics accumulated within microalgal cells disrupt the homeostasis of the reactive oxygen species in microalgal cells, thus increasing intracellular malondialdehyde (that causes cell peroxidative injury) and photosystem II activity (an indicator for microalgal photosynthetic activity); (3) microalgae cells activate self-protection mechanism to increase the production of intracellular photosynthetic pigments, antioxidants and carotenoids for protection against antibiotics-induced toxic effects, thus guaranteeing normal metabolism and development (Xiong et al., 2017).

It has been shown that adding exogenous phytohormones such as gibberellins (GAs) to the culture medium significantly increased the activities of glutathione peroxidase and total superoxide dismutase in microalgal cells, thus increasing the resistance of the cells under antibiotic stress. The GAs treatment also enhanced the removal of sulfamethoxazole (SMM) by *C. vulgaris* (Yang et al., 2023). These studies suggest that exogenous GAs supplementation can efficiently enhance microalgal removal of antibiotics. The present study aimed to determine the maximal removal efficiency of the seven commonly used antibiotics using three different bacteria-microalgae-fungi symbionts under different concentrations of GAs treatment.

2. Methods and materials

2.1. Microalgae, bacteria and fungi

2.1.1. Microalgae culture

C. vulgaris (FACHB-8) was provided by the Wuhan Institute of Hydrobiology, Chinese Academy of Sciences in Wuhan, China. The *C. vulgaris* was cultured in blue-green medium (BG11) for 7 days at 25 ± 2 °C under constant cool-white Light Emitting Diode (LED) light at the 200 μmol m^{-2}s^{-1} photosynthetic photon flux density and a 12-h light/12-h dark cycle. The composition of the BG11 medium was as follows: $K_2HPO_4{\cdot}3H_2O$ (0.04 g/L), $MgSO_4{\cdot}7H_2O$ (0.075 g/L), $CaCl_2{\cdot}2H_2O$ (0.036 g/L), citric acid (0.006 g/L), ferric ammonium citrate (0.006 g/L), EDTA (0.001 g/L), $NaNO_3$ (1.5 g/L), Na_2CO_3 (0.02 g/L), and 1.0 mL trace metal mix A5 [H_3BO_3 (2.86 g/L), $MnCl_2{\cdot}4H_2O$ (1.81 g/L), $ZnSO_4{\cdot}7H_2O$ (0.222 g/L), $NaMoO_4{\cdot}2H_2O$ (0.39 g/L), $CuSO_4{\cdot}5H_2O$ (0.079 g/L), and $CoCl_2{\cdot}6H_2O$ (0.05 g/L)]. The pH of the medium was adjusted to 7.1.

2.2. 2. Endophytic bacterial culture

C. vulgaris culture solution (10 mL) was subjected to 10-min centrifugation at 8000 r min^{-1}, followed by washing with sterile water, and the resultant algal slurry was completely blended in sterile water (0.5 mL) prior to a 20-min grinding. After grinding, the solution (100 μL) was spread on the Luria Bertani (LB) solid medium and incubated for 48-h at 37 ± 1 °C in the dark. Sterile water was used as a control to test whether the bacterial strain S395-2 existed in our cultured algae (Xu et al., 2020).

2.2.1. Fungal culture

Fresh soil sample collected in Yushu, Qinghai, China, was dissolved in sterile water on an ultra-clean bench, and 5 mL of the solution was spread in potato dextrose agar (PDA) medium, followed by a 48-h

incubation under 25 ± 2 °C in the dark. Plate streaking was later conducted to purify colonies, and those with characteristic morphological features were chosen. A certain amount of fungal mycelia was cultured on the PDA medium, followed by re-streaking to obtain single colonies. Thereafter, single fungal colonies were cultured in potato dextrose water (PDW) medium and incubated at 25 ± 2 °C in the dark. Dominant strains that were resistant to staining and had antibiotic stress adaptability were selected for identification. Finally, fungus *C. rosea* was identified as the strain in line with the previous description (Nagaraj et al., 2023).

2.3. Establishment of different algae co-culturing systems

2.3.1. Strain 1: Microalgal monoculture

C. vulgaris monoculture was prepared as depicted in 2.1.1. Algae inoculation was completed in fresh BG11 medium (five-fold volume) after algal biomass growth entered the exponential phase. This process was repeated to obtain adequate *C. vulgaris* for subsequent assays.

2.3.2. Strain 2: Microalgae-endophytic bacteria-C. Rosea symbiont co-culture

The bacterial strain S395-2 was cultured in LB medium (10 mL, 2.0 × 10^7 cells mL^{-1}) to the logarithmic growth phase, as depicted in section 2.1.2, and the culture was centrifuged at 8000 r min^{-1}. Thereafter, the supernatant was discarded, and the cell pellets were washed with sterile water via centrifugation. After discarding the supernatant, the bacterial cells were suspended with the freshly prepared BG11 medium (5 mL). *C. vulgaris* solution (10 mL, 2.0 × 10^6 cells mL^{-1}) was centrifuged, and bacterial and algal solutions (5 mL each) were added to BG11 medium (40 mL) for incubation at 28 ± 1 °C.

2.3.3. Strain 3: Microalgae -endophytic bacteria- Ganoderma lucidum symbiont co-culture

Fungal solution (10 mL, 2.0 × 10^7 cells mL^{-1}) was cultured to the logarithmic growth phase, as depicted in section 2.1.3, and added into sterilized BG11 culture solution on an ultra-clean bench. *C. vulgaris* solution (10 mL, 2.0 × 10^6 cells mL^{-1}) was also added to the medium, and the mixture was placed on a constant-temperature horizontal light shaker (28 ± 1 °C, ×160 rpm) in the dark to build the algal symbiont. After 12-h, the filamentous spheres of *Ganoderma lucidum* (*G. lucidum*) had absorbed *C. vulgaris*, and the culturing condition was changed to a 12-h light/12-h dark cycle.

2.3.4. Strain 4: Microalgae-endophytic bacteria-Pleurotus pulmonarius symbiont co-culture

After acquiring the *C. vulgaris-Pleurotus pulmonarius* (*P. pulmonarius*) spheres, as mentioned in section 2.2.3, the spheres were washed thrice using sterile BG11 medium, followed by the addition of bacterial solution (10 mL, 2.0 × 10^7 cells mL^{-1}). The mixture was then kept in a sterile chamber under constant light and temperature conditions (light intensity of 120 μmol m^{-2} s^{-1}, light/dark cycle of 12-h/12-h, and temperature 28 ± 1 °C) and rotational speed of 160 rpm.

2.4. Photo-bioreactor

A photo-bioreactor was designed according to a previous study (Wang et al., 2023) using two 16.8-L mutually-connected glass jars (see supplementary material). Briefly, simulated swine wastewater to be treated was added (2.8 L) in the right-sided glass jar of the reactor, and the four microbial strains were added into the left glass jar for treatment experiments. After the treatment, the water sample was collected through the sampling port of the left glass jar, after which six white LED lamps (20 W/110 V, 200 μmol m^{-2} s^{-1}) were installed on the left glass jar. The light/dark ratio and temperature of the reaction were controlled at 12-h/12-h and 25 ± 2 ℃.

2.5. Simulated swine wastewater

The simulated swine wastewater contained 0.004 g/L $MgSO_4$, 0.0322 g/L KH_2PO_4, 0.432 g/L $NaH_2PO_4{\cdot}2H_2O$, 0.008 g/L $CaCl_2$, 1.506 g/L urea, 1.6 g/L glucose, and a final concentration of 1 mg/L of TC, OTC, Ciprofloxacin (CPFX), Norfloxacin (NFX), SMZ, SMX and SMM. The antibiotics, ((CAS No: 64–75-5), OTC (CAS NO: 2058–46–0), CPFX (CAS NO: 85721–33-1), NFX (CAS NO: 70458–96-7), SMZ (CAS NO: 68–35-9), SMX (CAS NO: 57–68-1), and SMM (CAS NO: 723–46-6)) were obtained from Shanghai Macklin Biochemical Co., Ltd, and were added at the same time in swine simulated water. Meanwhile, the biogas slurry had a pH of 6.87 ± 0.32, chemical oxygen demand (COD) of 2739.18 ± 59.41 mg/L, total nitrogen (TN) of 598.72 ± 28.37 mg/L, and total phosphorus (TP) of 78.07 ± 5.16 mg/L. The GAs reagent was obtained from 159 MACKLIN (Shanghai, China) and was added to the medium after sterilization to prevent denaturation.

2.6. Experimental procedures

Each experiment was conducted in triplicate, and the experiment duration was 10 days. The initial biomass of the four strains within the photo-bioreactor was 103.82 ± 7.64 mg/L. The culture system used to purify swine wastewater was treated with 1 mg/L of the antibiotics. To compare the antibiotic removal capacity and growth cycle of algal symbionts, we collected water samples on days 3, 7 and 10 to measure the antibiotic removal efficiency (an indicator of performance), mean daily productivity, growth rate, chlorophyll *a* (CHL-a) content, and fluorescence transient test parameters. The antibiotic removal performance of the four algal symbionts was analyzed to determine the suitable antibiotic removal approach. The temperature was set at 25.0 ± 2 ℃, and the white LED lamp on the left-side glass jar of the photo-bioreactor was used to provide the light source (14-h light:10-h dark cycle; light intensity of 200 μmol m^{-2} s^{-1}).

2.7. Analytical methods and definitions

2.7.1. Cell growth analysis

The samples (10 mL) were centrifuged, washed and filtered with the 0.45-μm disposable PES filter membrane. Thereafter, the sample-containing PES filter membrane and the one with the control (sterile water) were subjected to 6-h oven-drying at 105 °C and weighed using an electronic balance to obtain the dry weight (DW). The DW was determined by obtaining the differences between the filter weights before and after filtration. Eq. (1) was utilized to determine the strain-specific growth rate (U, d^{-1}), while Eq. (2) was employed to calculate the average daily productivity (P, g/L d^{-1}).

$U = (\ln DW_i - \ln DW_0)/t_i \ln DW_i = Ut_i + \ln DW_0$ (1).

$P = (DW_i - DW_0)/(t_i - t_0)$ (2).

Where, DW_i and DW_0 represent sample biomass on day i (t_i, d) after the experiment and day 0 before the experiment (t_0, d), respectively.

2.7.2. Chlorophyll a (CHL-a) content determination

Strain solution (4 mL) was collected and centrifuged for 10 min at 8000 rpm to obtain the precipitate, which was dissolved in 90 % v/v acetone (4 mL) by mixing using a vortex. Thereafter, the mixture was incubated for 24-h at 4 ℃ in the dark, followed by a 10-min centrifugation (×8000 rpm). The absorbance of the supernatant was measured using a UV–Vis spectrophotometer at 630 nm, 645 nm, 663 nm, and 750 nm (OD_{663}, OD_{750}, OD_{645}, and OD_{630}). Meanwhile, 90 % acetone was the blank control. The CHL-a content, ρ (CHL-a), of the strains, was determined using Eq. (3):

$$\rho(\text{CHL-a}) = 11.64 \times (OD_{663nm} - OD_{750nm}) + 0.10 \times (OD_{630nm} - OD_{750nm}) - 2.16 \times (OD_{645nm} - OD_{750nm}) \quad (3)$$

2.7.3. Photosynthetic activity analysis

The AquaPen handheld chlorophyll fluorescence meter (JQ977-FluorPen FP110) was utilized in each experiment. The samples (4 mL) were collected, put into the cuvette, and covered with aluminium foil to achieve a 5-min dark adaptation. The samples were then transferred into a chlorophyll fluorescence tester to measure the fast chlorophyll fluorescence kinetics (OJIP) parameter. The F_V/F_M, PI_{ABS}, Φ_{EO}, and Ψ_O parameters, representing the absorbed light energy-based performance index, electron transfer quantum yield, and electron transfer efficiency per captured exciton energy, respectively, were analyzed.

2.7.4. Antibiotics concentration analysis

The water samples (4 mL) were centrifuged for 10 min at × 8000 rpm, and the supernatant was filtered through a 0.45-μm PES filter membrane, followed by antibiotic concentration analysis of the resultant filtrate. The antibiotic concentration was determined using HPLC under the following liquid-phase conditions: ZORBAX SB-C18 column (5 μm, 250 mm × 4.6 mm); column temperature, 35 ℃; mobile phase for elution: acetonitrile: 0.01 M sodium dihydrogen phosphate = 20 %: 80 %; injection volume, 20 μL; flow rate, 1 mL min^{-1}. The absorbance values of (OTC, TC), (NFX, CPFX) and (SMZ, SMX and SMM) were analyzed with a spectrophotometer at 214 nm, 277 nm and 272 nm, respectively, with pure water as the reference. Eq. (4) was applied to calculate antibiotic removal (R):

$$R = (C_0 - C_i)/C_0 \times 100 \quad (4)$$

Where, C_0 and C_i represent antibiotic concentrations (mg/L) before and after treatment, respectively.

2.8. Data processing and statistical analysis

Data were presented as mean ± standard deviation from at least three independent assays. SPSS software 19.0 (IBM Corp., Armonk, NY, USA) was utilized for statistical analysis. Correlation tests were performed using Duncan's multiple comparison test to examine the different effects of altering the initial antibiotic concentration on strain growth, antibiotic removal and photosynthetic activity. $P < 0.05$ represented statistical significance.

3. Results and discussion

3.1. Growth of the four co-culture systems under different GAs treatment concentrations

The effects of four different concentrations of GAs treatments (0, 20, 50, and 80 mg/L) on the growth of the four bio symbionts in the simulated wastewater supplemented with seven antibiotics at the initial concentration of 1 mg/L were determined at three time-points (day 3, day 7 and day 10). The mean daily productivity of the co-culture systems changed with time and GAs concentrations (Fig. 1). For mono-microalgae culture, the 50 mg/L of GAs treatment increased the mean daily production at all three time-points tested compared to the 20 mg/L GAs treatment whereas 80 mg/L GAs had minor effects on the mean daily production compared with 20 and 50 mg/L GAs (Fig. 1a). Moreover, 50 mg/L GAs had the highest mean daily production at the 7th day time-point (Fig. 1a). A similar change trend in the mean daily production was observed in the three symbionts treated with different GAs concentrations (Fig. 1 b-d). Interestingly, among the three symbionts, Strain 2 had the highest mean daily productivity at the 7th-day time-point (Fig. 1a-d).

These results might be explained by strain growth features under antibiotic stress (Zambrano et al., 2023). On day 7 under the 50 mg/L GAs treatment with the 1 mg/L initial antibiotic concentration, the mean daily productivity of the four systems followed the order of Strain 2 > Strain 3 > Strain 4 > Strain 1, which reached 0.229 ± 0.017, 0.195 ± 0.015, 0.175 ± 0.014, and 0.171 ± 0.012 g/L d^{-1}, respectively. In addition, the responses of the strain cells to different GAs concentrations in the culture were probably influenced by the microalgal cell growth rate within the symbiont.

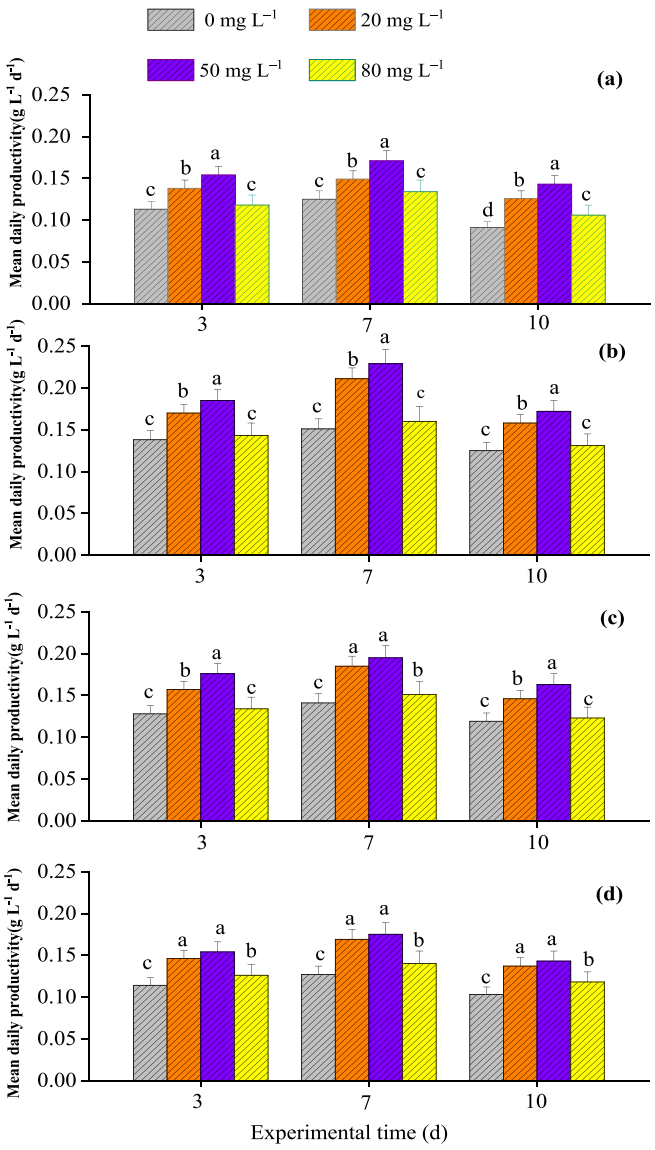

Fig. 1. Mean daily productivity of the four selected strains under three different concentration of GAs treatment for 3 days, 7 days and 10 days. Treatment 1 is only *C. vulgaris*, Treatment 2 is *C. vulgaris*- S395-2-*C. rosea* co-culturing, (c) Treatment 3 is *C. vulgaris*- S395-2-*G. lucidum* co-culturing, (d) Treatment 4 is *C. vulgaris*- S395-2-*P. pulmonarius* co-culturing.

The effects of the different GAs concentrations on the growth rates of the four strains followed the order: 50 mg/L > 20 mg/L > 80 mg/L > 0 mg/L (see supplementary material). However, the growth rate of the four treatments had the following order: treatment 2 > treatment 3 > treatment 4 > treatment 1. Thus, 80 mg/L of the GAs treatment did not significantly affect the growth rate of the four treatments tested. GAs concentrations of 20 and 50 mg/L significantly promoted growth ($P < 0.05$), with 50 mg/L GAs exhibiting the highest growth promotion effects ($P < 0.05$).

High-concentration antibiotics can change redox conditions, photosynthesis, intracellular biochemistry, and microalgal growth patterns, which can modulate biotransformation and antibiotic absorption (Yu et al., 2022). These findings were consistent with a previous study, which reported that 1–50 mg/L of the TC treatment decreased the algal

system biomass relative to blank control (Chu et al., 2023). Conversely, at the initial concentration of < 50 mg/L, OTC enhanced the microglial growth but markedly suppressed the microglial development at > 100 mg/L (Wu et al., 2022). These discrepancies might be attributed to different antibiotic types, strain species in the culture and symbiont conditions used in the studies.

3.2. Chl-a contents of the four culture systems under different GAs treatment concentrations

To verify how the GAs treatments affected the growth of the four strains, we measured the CHL-a contents of the four strains from every system under different GAs treatment concentrations. The CHL-a content was increased after treatment with 20 and 50 mg/L GAs relative to the no-GAs supplementation control (Fig. 2). In addition, the CHL-a contents of the four strains within the bacteria-microalgae-fungi symbionts and microalgal monoculture initially increased and later decreased under 80 mg/L GAs treatment, compared with 20 and 50 mg/L GAs treatments (Fig. 2). Besides, the highest CHL-a content was detected in every system and at time-point with the 1 mg/L initial concentration of the seven different antibiotics.

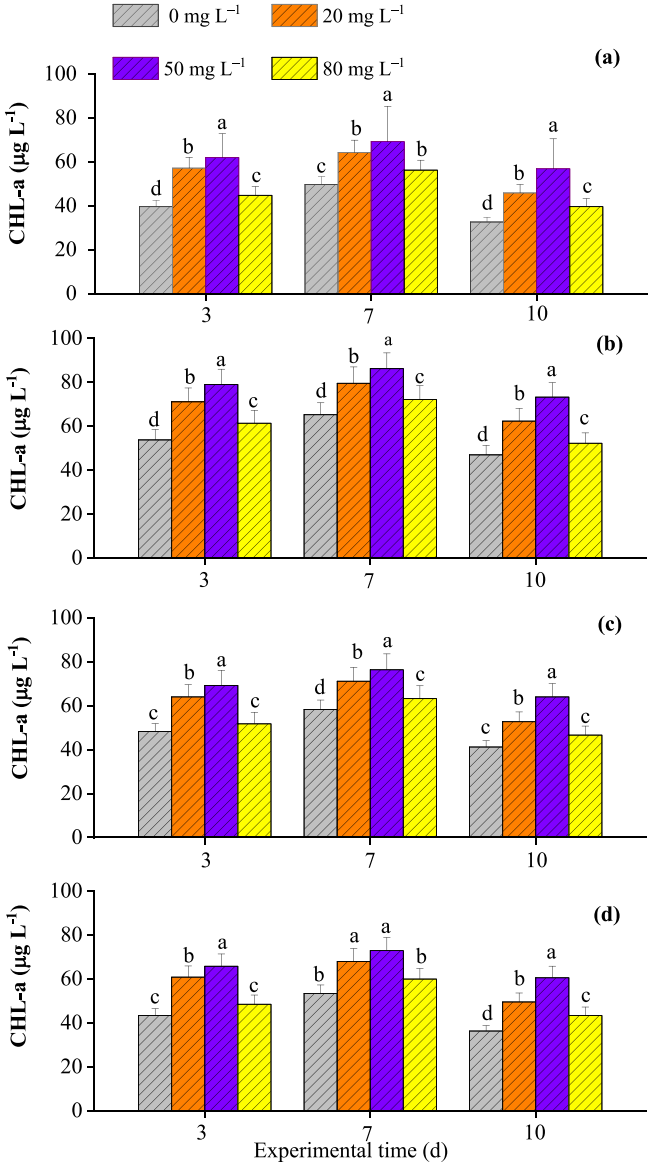

Fig. 2. The CHL-a content of the four selected strains under three different GAs concentrations treatments for 3 days, 7 days and 10 days. Treatment 1 is only *C. vulgaris*, Treatment 2 is *C. vulgaris*- S395-2-*C. rosea* co-culturing, Treatment 3 is *C. vulgaris*- S395-2-*G. lucidum* co-culturing, (d) Treatment 4 is *C. vulgaris*-S395-2-*P. pulmonarius* co-culturing.

Changes in the CHL-a contents of the four strains exhibited a similar trend under the same initial antibiotics concentrations, which peaked on day 7 during the experiment (Fig. 2). The CHL-a contents of every system followed the order: Strain 2 > Strain 3 > Strain 4 > Strain 1, which reached 86.17 ± 7.16 μg/L, 76.33 ± 7.2 μg/L, 72.85 ± 5.91 μg/L, and 69.17 ± 16.07 μg/L, after 7 days of treatment with 1 mg/L antibiotics, respectively.

The CHL-a content of Strain 2 increased by 1.12–1.24 folds relative to the other three systems treated with 50 mg/L GAs. This suggested the higher tolerance of the bacteria-microalgae-fungi three-phase symbiont to multiple antibiotics compared to microalgae monoculture and two-phase co-cultures (Sharma et al., 2021; Yu et al., 2022).

3.3. Photosynthetic activities of the four culture systems under different GAs concentrations

Photosynthesis is an important metabolic activity for autotrophic organisms; however, antibiotics may influence photosynthesis by disrupting photosystem I (PSI) and photosystem II (PSII), thus decreasing protein and pigment biosynthetic efficiency (Li et al., 2023). The photosynthetic activities of the four strains within the *C. vulgaris* monoculture system and the three algal symbioses under different GAs concentrations were measured. The F_V/F_M ratio is mostly obtained based on CHL-a molecules in the PSII reaction centers of strain cells, which is usually adopted to indicate environmental stress (Bi et al., 2012). The GAs treatments and untreated control showed significant differences in the same symbiont systems (Table 1), with 50 mg/L GAs having the most prominent effect on daily production. The F_V/F_M value of Strain 2 treated with 50 mg/L GAs and 1 mg/L of the antibiotics was 0.89 ± 0.09, one-fold higher than the F_V/F_M value of strain 1 (0.43 ± 0.04) of microalgal monoculture (Table 1). The decrease in the F_V/F_M values was observed when the strain treated with 80 mg/L GAs was compared with that treated with 50 mg/L GAs (Table 1).

Similar results were previously reported whereby the fluorescence intensity of an algal system showing a low CPFX concentration (10 mg/L) was the opposite of that exhibiting a high CPFX concentration (50 mg/L) (Chu et al., 2023). It was speculated that a reversible impairment of the maximal photochemical quantum yield of strain cells caused by antibiotics at an appropriate concentration might be recovered during the late incubation stage. This was supported by the results that PSII reaction centers in *Pseudomonas aeruginosa* strains exhibited resistance to TC, while their F_V/F_M values were reversed following low-concentration antibiotic treatments (Yang et al., 2013). This suggested that strain photosynthesis is mitigated following low-concentration antibiotic treatment and the non-stressful products in the strains gradually degrade antibiotics. Besides, the high-concentration antibiotic treatment of 1 mg/L significantly inhibited strain photosynthetic activity.

The absorption performance index of light energy (PI_{ABS}) of the four strains increased under three different GAs treatments (20 mg/L, 50 mg/L and 80 mg/L) compared with the untreated monoculture, with 50 mg/L GAs having the highest effect (Table 1). Besides, mechanoenergy captured reduced the electron transfer efficiency (Ψ_O) and electron transfer quantum yield (Φ_{EO}), which in turn reduced the maximal optical efficiency (F_V/F_M) of the strains.

Compared to the photosynthetic activity parameters for 1 mg/L antibiotic-treated free microalgal cells, the levels of these parameters increased in the bacteria-microalgae-fungi symbiont, with the highest values being observed in the *C. vulgaris*-endophytic bacterial (S395-2)-*C. rosea* three-phase symbiont. This finding indicated the highest optical efficiency, the greatest performance index of light energy absorption, most reactive reaction centers within the photosystem, the greatest

Table 1
The fluorescence data obtained via OJIP test of the four selected strains in the periods time.

	Treatment 1	Treatment 2	Treatment 3	Treatment 4
GAs concentrations	F_V/F_M			
0 mg/L	$0.26^{d} \pm 0.02$	$0.43^{d} \pm 0.04$	$0.37^{c} \pm 0.04$	$0.34^{c} \pm 0.03$
20 mg/L	$0.51^{b} \pm 0.05$	$0.72^{b} \pm 0.07$	$0.61^{b} \pm 0.06$	$0.57^{b} \pm 0.06$
50 mgL^{-1}	$0.62^{a} \pm 0.06$	$0.89^{a} \pm 0.09$	$0.77^{a} \pm 0.08$	$0.65^{a} \pm 0.07$
80 mg/L	$0.35^{c} \pm 0.04$	$0.61^{c} \pm 0.06$	$0.43^{c} \pm 0.04$	$0.41^{c} \pm 0.04$
	PI_{ABS}			
0 mg/L	$3.52^{d} \pm 0.33$	$4.96^{d} \pm 0.48$	$4.24^{c} \pm 0.39$	$3.87^{c} \pm 0.35$
20 mg/L	$6.23^{b} \pm 0.59$	$6.61^{b} \pm 0.63$	$6.63^{a} \pm 0.64$	$5.79^{a} \pm 0.56$
50 mgL^{-1}	$6.92^{a} \pm 0.68$	$7.24^{a} \pm 0.71$	$6.82^{a} \pm 0.65$	$6.41^{a} \pm 0.62$
80 mg/L	$4.91^{c} \pm 0.47$	$5.73^{c} \pm 0.55$	$5.49^{b} \pm 0.51$	$5.32^{b} \pm 0.49$
	Ψ_O			
0 mg/L	$0.52^{d} \pm 0.05$	$0.69^{d} \pm 0.07$	$0.56^{d} \pm 0.06$	$0.52^{b} \pm 0.05$
20 mg/L	$0.82^{b} \pm 0.08$	$0.93^{b} \pm 0.09$	$0.83^{b} \pm 0.08$	$0.76^{b} \pm 0.08$
50 mgL^{-1}	$0.93^{a} \pm 0.09$	$1.18^{a} \pm 0.10$	$0.92^{a} \pm 0.09$	$0.87^{a} \pm 0.09$
80 mg/L	$0.71^{c} \pm 0.07$	$0.86^{c} \pm 0.09$	$0.75^{c} \pm 0.07$	$0.71^{c} \pm 0.08$
	Φ_{EO}			
0 mg/L	$0.29^{c} \pm 0.03$	$0.53^{d} \pm 0.05$	$0.42^{d} \pm 0.04$	$0.37^{d} \pm 0.04$
20 mg/L	$0.42^{b} \pm 0.04$	$0.82^{b} \pm 0.08$	$0.69^{b} \pm 0.07$	$0.58^{b} \pm 0.06$
50 mgL^{-1}	$0.65^{a} \pm 0.07$	$0.96^{a} \pm 0.09$	$0.84^{a} \pm 0.08$	$0.75^{a} \pm 0.07$
80 mg/L	$0.34^{b} \pm 0.03$	$0.63^{c} \pm 0.06$	$0.51^{c} \pm 0.05$	$0.46^{c} \pm 0.05$

Note: The data in Table 1 were expressed as mean ± SD (n = 3). Values with different.
superscript letters indicate a significant difference at $P < 0.05$.

electron transfer quantum field, and the highest photosynthetic activity of strains in the *C. vulgaris*-endophytic bacterial (S395-2)-*C. rosea* three-phase symbiont. This might be because microalgal growth enhanced the actions of fungal and bacterial strains within this three-phase symbiont, thus alleviating the inhibition of antibiotics on *C. vulgaris*. Besides, a previous study showed that such bacteria-microalgae-fungi symbiont enhanced the microalgae-mediated light energy absorption and electron transfer efficiency (Dong et al., 2022a).

3.4. Antibiotics removal efficiencies of the four culture systems under different GAs concentrations

The mean removal efficiencies of TC, OTC, CPFX, NFX, SMZ, SMX, and SMM, which had initial concentrations of 1 mg/L, by the four systems were determined for 10 days (Table 2). Different GAs concentrations significantly affected the removal efficiencies of the 7 antibiotics ($P < 0.5$), with 50 mg/L GAs exhibiting the highest removal efficiencies of the 7 antibiotics. The antibiotic removal effect of the algal symbionts was closely related to their growth and photosynthetic performance. Adding GAs at the appropriate concentration could increase the specific growth rate and daily production of the algal symbionts and enhance their photosynthetic performance parameters to a certain extent. However, high concentrations of GAs (e.g., 80 mg/L) limited the growth, photosynthesis and antibiotic removal performance of the algal symbionts. This may be because the high concentration of GAs increased the content of free radical species in algal cells (but not to enough levels to

Table 2
Mean values ± SD of the removal efficiency of the four selected strains at various GAs concentrations treatments.

GAs concentrations	Treatment 1	Treatment 2	Treatment 3	Treatment 4
	TC Removal efficiency (%)			
0 mg/L	$82.6^{b} \pm 8.0$	$87.1^{c} \pm 6.4$	$84.4^{c} \pm 6.0$	$83.4^{c} \pm 7.0$
20 mg/L	$90.4^{a} \pm 8.7$	$94.8^{ab} \pm 3.2$	$93.2^{ab} \pm 4.2$	$92.3^{ab} \pm 6.2$
50 mgL^{-1}	$92.4^{a} \pm 6.0$	$96.0^{a} \pm 1.4$	$96.5^{a} \pm 2.0$	$95.3^{a} \pm 6.4$
80 mg/L	$87.0^{a} \pm 8.4$	$92.4^{bc} \pm 5.5$	$89.6^{bc} \pm 7.2$	$88.3^{b} \pm 6.2$
	OTC Removal efficiency (%)			
0 mg/L	$76.4^{c} \pm 7.0$	$81.5^{b} \pm 7.9$	$78.4^{c} \pm 7.1$	$77.4^{c} \pm 7.0$
20 mg/L	$81.7^{b} \pm 7.7$	$88.5^{a} \pm 8.4$	$85.2^{ab} \pm 8.2$	$83.6^{b} \pm 7.3$
50 mgL^{-1}	$86.4^{a} \pm 8.1$	$92.1^{a} \pm 4.9$	$89.5^{a} \pm 8.2$	$88.8^{a} \pm 8.0$
80 mg/L	$79.3^{bc} \pm 6.3$	$83.5^{b} \pm 8.0$	$81.4^{bc} \pm 7.8$	$80.5^{bc} \pm 7.1$
	CPFX Removal efficiency (%)			
0 mg/L	$32.2^{c} \pm 2.7$	$42.9^{c} \pm 3.8$	$38.8^{c} \pm 3.4$	$36.3^{c} \pm 3.0$
20 mg/L	$39.6^{b} \pm 2.9$	$46.7^{b} \pm 4.3$	$45.9^{b} \pm 3.9$	$42.3^{b} \pm 3.2$
50 mg/L	$45.1^{a} \pm 3.4$	$48.7^{a} \pm 5.1$	$52.4^{a} \pm 4.5$	$49.5^{a} \pm 4.4$
80 mg/L	$37.3^{b} \pm 2.8$	$46.3^{bc} \pm 4.0$	$43.5^{b} \pm 4.0$	$39.7^{bc} \pm 3.1$
	NFX Removal efficiency (%)			
0 mg/L	$22.5^{b} \pm 1.6$	$30.0^{d} \pm 2.5$	$26.6^{d} \pm 2.5$	$24.7^{c} \pm 2.0$
20 mg/L	$29.5^{a} \pm 2.0$	$32.6^{b} \pm 2.8$	$31.3^{b} \pm 3.0$	$30.4^{ab} \pm 2.5$
50 mg/L	$31.5^{a} \pm 2.4$	$34.6^{a} \pm 2.9$	$34.3^{a} \pm 3.1$	$33.9^{a} \pm 3.1$
80 mg/L	$27.2^{a} \pm 1.6$	$30.1^{c} \pm 2.8$	$29.7^{c} \pm 2.6$	$28.2^{bc} \pm 2.2$
	SMZ Removal efficiency (%)			
0 mg/L	$41.3^{c} \pm 3.3$	$52.2^{c} \pm 5.0$	$49.8^{c} \pm 4.4$	$45.7^{c} \pm 4.0$
20 mg/L	$45.0^{c} \pm 3.7$	$59.5^{b} \pm 5.2$	$56.3^{b} \pm 5.4$	$51.9^{b} \pm 4.4$
50 mg/L	$52.7^{b} \pm 4.3$	$61.0^{a} \pm 5.5$	$60.2^{a} \pm 6.0$	$59.5^{a} \pm 5.0$
80 mg/L	$42.6^{a} \pm 3.5$	$57.6^{b} \pm 5.1$	$54.2^{b} \pm 4.7$	$47.4^{bc} \pm 4.1$
	SMX Removal efficiency (%)			
0 mg/L	$48.4^{c} \pm 3.7$	$57.0^{b} \pm 5.3$	$53.6^{c} \pm 4.6$	$51.8^{b} \pm 4.2$
20 mg/L	$53.7^{b} \pm 4.2$	$58.4^{b} \pm 5.7$	$56.7^{b} \pm 4.8$	$54.9^{b} \pm 4.8$
50 mgL^{-1}	$57.1^{a} \pm 5.5$	$63.7^{a} \pm 5.6$	$62.4^{a} \pm 5.4$	$59.1^{a} \pm 5.3$
80 mg/L	$50.2^{bc} \pm 4.8$	$57.0^{b} \pm 5.6$	$55.6^{bc} \pm 4.6$	$52.4^{b} \pm 4.3$
	SMM Removal efficiency (%)			
0 mg/L	$37.4^{c} \pm 3.3$	$48.4^{c} \pm 4.4$	$44.4^{c} \pm 4.0$	$40.5^{c} \pm 3.7$
20 mg/L	$43.0^{b} \pm 4.1$	$51.7^{ab} \pm 5.0$	$49.6^{ab} \pm 4.4$	$45.8^{b} \pm 4.3$
50 mg/L	$48.6^{a} \pm 4.4$	$54.8^{a} \pm 4.9$	$53.1^{a} \pm 5.2$	$52.2^{a} \pm 5.0$
80 mg/L	$40.2^{bc} \pm 3.7$	$50.7^{b} \pm 4.6$	$46.7^{bc} \pm 4.1$	$43.8^{bc} \pm 3.2$

Note: Values with different superscript letters indicate a significant difference at $P < 0.05$ according to the Duncan's multiple range tests for the same strain under different treatments.

cause serious cell damage), which in turn increased the physiological, biochemical and metabolic activities and compensatory proliferation of algal cells, limiting their stimulatory effect on algal cell growth (Yang et al., 2023). Strain 2 had the best removal efficiency among the four symbionts used, and the removal efficiency rankings for strain 2 were as follows: TC > OTC > CPFX > NFX > SMX > SMZ > SMM.

It has been shown that antibiotics mainly undergo bioadsorption, bioaccumulation, and biodegradation sequentially in algal symbionts (Eheneden et al., 2023). In *Paraclostridium* sp., CPFX was mainly removed by altering the CPFX initial concentration (0.1–20 mg/L), showing the main role of biosorption in removing antibiotics with lower initial concentrations (Fang et al., 2021). However, as the initial

concentration increased, biotransformation was the predominant antibiotic removal mechanism. We found that 20 mg/L CPFX decreased the biotransformation efficiency to 1975.7 ± 109.1 μg g^{-1}-cell dry weight h^{-1}, consistent with other studies (Chu et al., 2023). These findings may be because biodegradation is easily affected by substrate limitation thresholds, which depend on target pollutant concentration. Biosorption is a prerequisite for bioaccumulation (Norvill et al., 2016). In this study, the structure, molecular weight, polar bond number, and type of antibiotics affected the biosorption process of the algal symbionts.

TC, OTC, CPFX, NFX, SMZ, SMX, and SMM antibiotics utilized in the present study had chemical molecular weights of 444.43, 496.89, 331.34, 319.33, 250.28, 278.33, and 253.28, respectively. Based on their antibiotic removal efficiencies, the four strains could easily adsorb large-molecular-weight antibiotics, similar to previous studies (Hena et al., 2020; Liu et al., 2022). Additionally, polar bond number and antibiotic type affected the binding of algal symbiont cells to the antibiotics. Relative to SM_2 (1 amine group-NH_2, 1 sulfonyl group-SO_2-) and CPFX (2 carbonyls = O, 1 hydroxyl-OH), OTC has more types and number of reactive functional groups (namely, 1 amine group-NH_2, 6 hydroxyl-OH, and 3 carbonyls = O) in each molecular structure, and these contribute to OTC adsorption and removal (Tang et al., 2022).

Different strains had different antibiotic removal efficiencies of the 7 seven antibiotics, with initial concentrations of 1 mg/L, under different concentrations of exogenous GAs supplementation. Strain 2 (*C. vulgaris*-endophytic bacterial (S395-2)-*C. rosea* symbiont) exhibited the highest removal efficiencies of the 7 antibiotics under the 50 mg/L GAs treatment on day 7. The average removal rates of the 7 antibiotics by Strain 2 were 97.0 ± 2.8 % for TC (Fig. 3a), 92.7 ± 5.2 % for OTC (Fig. 3b), 49.6 ± 4.4 % for CPFX (Fig. 4a), 34.8 ± 2.2 % for NFX (Fig. 4b), 61.4 ± 5.5 % for SMZ (Fig. 5a), 64.8 ± 5.8 % for SMX (Fig. 5b), and 57.0 ± 4.4 % for SMM (Fig. 5c). This result differs from the results of the previous study,

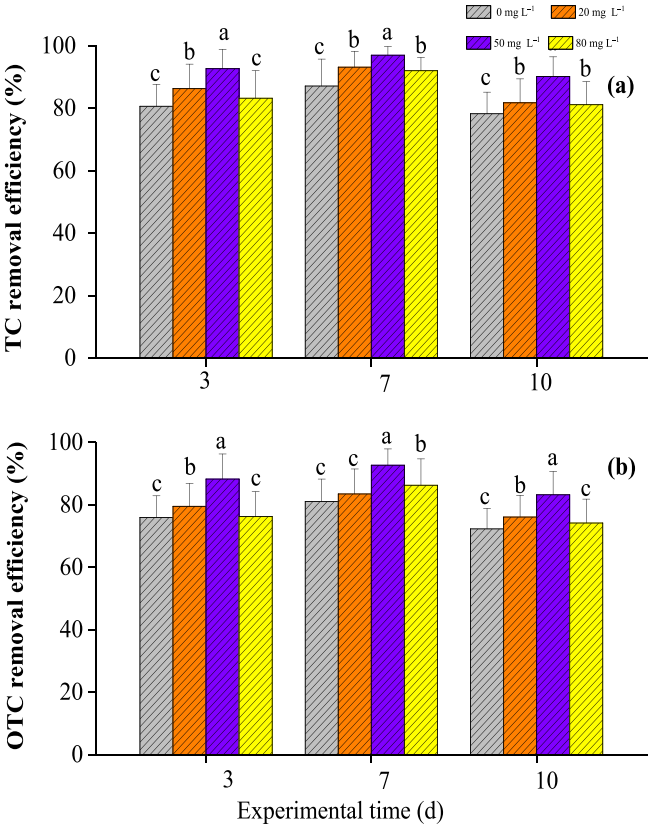

Fig. 3. The time-course tetracycline antibiotics removal efficiency using Strain 2 under different initial concentrations of GAs treatment. (a) OTC removal efficiency, (b) TC removal efficiency.

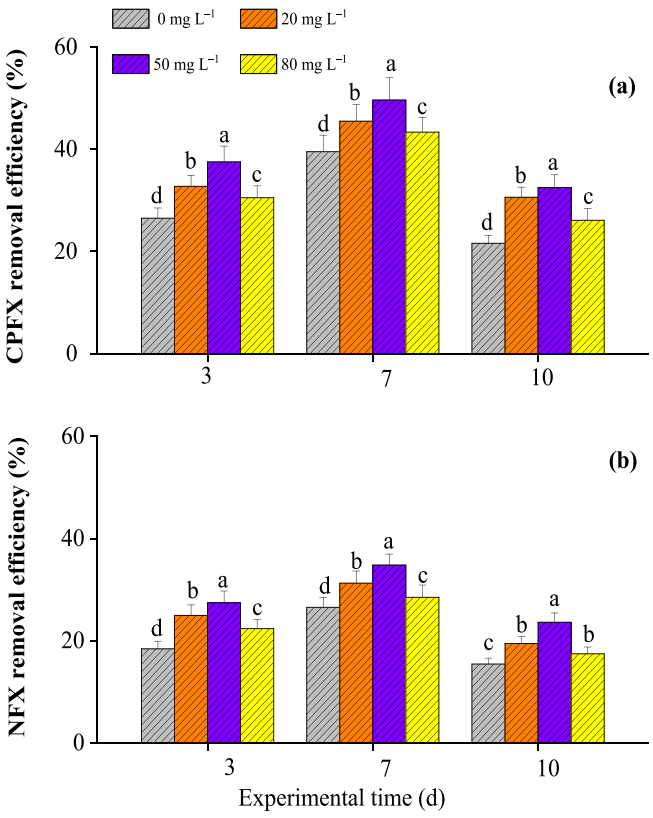

Fig. 4. The time course sulfonamides antibiotics removal efficiency using Strain 2 under different initial concentrations of GAs treatment. (a) CPFX removal efficiency, (b) NFX removal efficiency.

which showed that the removal of OTC by algal symbionts could reach 99.4 ± 0.5 % at an initial antibiotic concentration of 0.25 mg/L (Wang et al., 2023). This is probably due to the fact that the removal effect of antibiotics is related to the constructive composition of the algal symbionts and the concentration of antibiotics. Lower concentrations of antibiotics stimulated the growth of the algal symbionts, while higher concentrations inhibited their growth.

The antibiotic removal capacity of diverse symbionts is probably associated with two factors: (1) different extracellular polymeric substances (EPS) contents that impact antibiotic transfer across biofilms. A previous study reported that after antibiotic treatment, biofilm enzyme activities and respiration rate with/without low EPS content declined (Zhao et al., 2023). Moreover, bacteria-microalgae-fungi three-phase symbiont could generate an increased amount of EPS compared with two-phase symbiont and microalgae monoculture, contributing to antibiotics removal (Wang et al., 2023). (2) Differences in the stability of symbiotic structures, which mainly accounted for the higher antibiotic removal efficiency of *C. vulgaris*-endophytic bacterial (S395-2)-*C. rosea* symbiont than those reported previously. The fungus used in strain 2 was the most dominant (*C. rosea*), and it formed algal spheres with *C. vulgaris* that had better growth, photosynthesis and antibiotic removal properties than the other two fungi. The possible reasons for this could be better synchronization of the mycelial growth with that of *C. vulgaris*, better uniformity in the sizes of algal spheres formed, better adaptation to various antibiotics, and better resistance to antibiotics stress.

The efficiencies of microalgae-bacteria systems in removing antibiotics of the same types and concentrations (20–1000 μg/L) were 43 %–100 % (Eheneden et al., 2023). The sulfamethazine removal efficiency of the microalgae-fungus system was reported to be 58.71–67.91 % (Li et al., 2022). Bacteria-microalgae-fungi symbiotic systems mutually contribute to the conversion of organic and inorganic matter (Dong et al., 2022b). Fungal hyphae can tightly bind microalgae via potent

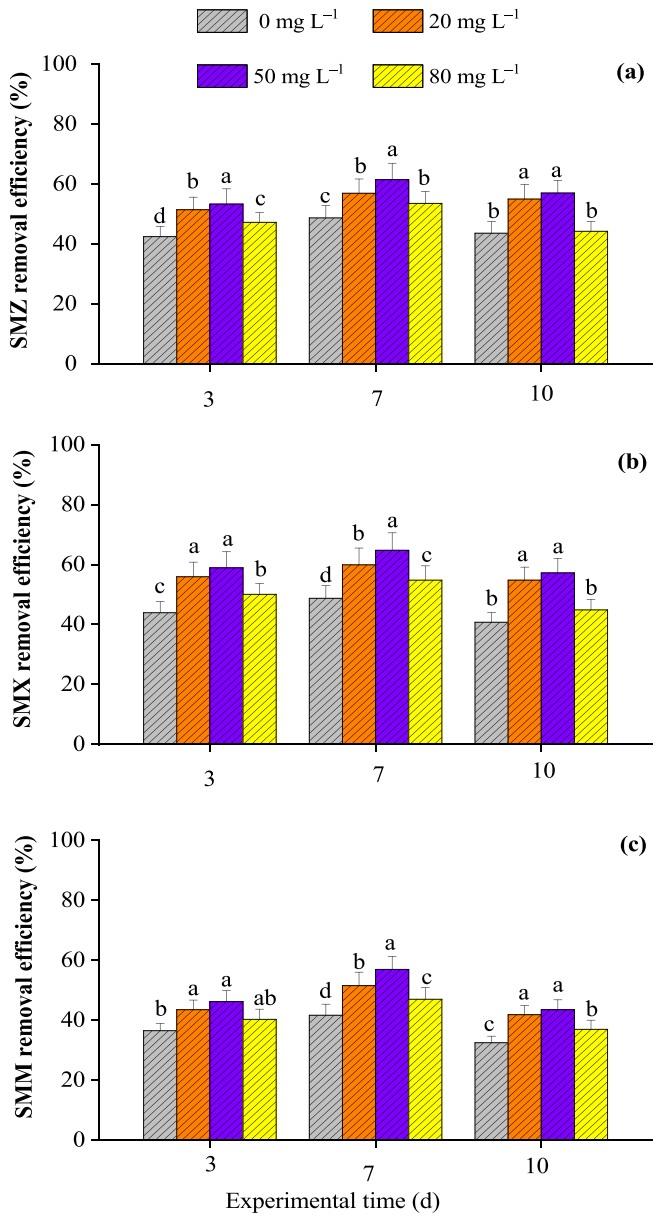

Fig. 5. The time course sulfadiazine antibiotics removal efficiency using Strain 2 under different initial concentrations of GAs treatment. (a) SMZ removal efficiency, (b) SMX removal efficiency, (c) SMM removal efficiency.

extracellular polysaccharide adhesion, adsorption energy, surface protein interactions, and electrostatic neutralization, whereas endophytic bacteria can better increase the microalgal and fungal binding capacity to construct symbiotic structures with higher stability (Wei et al., 2023). This ensures efficient protection of the microalgae against exposure to pollutants like antibiotics. The antibiotic removal efficiencies of the different systems tested in this study were low on the 3rd day, suggesting that these strains did not interact well with or exchange substrates after exposure to antibiotics at the beginning of the experiment, leading to the low removal performance in the early stage. After combining the CHL-a content with OJIP photosynthetic activity parameters, the antibiotics removal efficiencies of algal symbionts suddenly increased on the 7th day, probably due to biodegradation (Fig. 3- Fig. 5).

However, the antibiotic removal markedly reduced on the 10th day (Fig. 3- Fig. 5). Two possible mechanisms may explain this phenomenon: 1) the decreased effect of antibiotics-induced stress had a lesser impact on algal symbionts, and as a result, the symbiont systems might have secreted a lower amount of EPS upon the decreased antibiotic toxicity (Wang et al., 2018). 2) The antibiotic concentration decreased if the antibiotic-to-algal symbiont ratio, which impacted antibiotic degradation, was changed (Cheng et al., 2023). Therefore, the efficiency of antibiotic reduction in wastewater can be enhanced by regulating the EPS content in the symbiotic system or by changing the addition ratio of algal symbionts.

4. Conclusions

The *C. vulgaris*-endophytic bacterial (S395-2)-*C. rosea* symbiont treated with 50 mg/L GAs exhibited the highest growth, photosynthetic efficiency and removal efficiency of the 7 antibiotics. This was attributed to a more stable algal symbiosis induced by suitable concentrations of GAs and the secretion of EPS to resist unfavorable antibiotic stress. The GAs-treated bacteria-microalgae-fungi symbiont performed much better than the untreated symbiont and showed higher stress resistance and suitability for treating antibiotics wastewater. However, considering the heterogeneities of actual wastewater, further pilot-scale studies are needed to verify the adaptability and antibiotic removal efficiency of symbionts during practical wastewater treatment.

CRediT authorship contribution statement

Jun Liu: Writing – review & editing, Writing – original draft. **Zhengfang Wang:** Writing – review & editing, Writing – original draft, Investigation. **Chunzhi Zhao:** Methodology, Formal analysis, Data curation. **Bei Lu:** Methodology, Formal analysis. **Yongjun Zhao:** Supervision, Funding acquisition, Conceptualization.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.130182.

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