Micropropagation of eucheumatoids using liquid extracts from the brown algae Ascophyllum nodosum (Fucales) and Laminaria digitata (Laminariales)

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Abstract

Eucheuma denticulatum and *Kappaphycus alvarezii*, two tropical red seaweeds, are two of the most commercially important, cultivated carrageenophytes in Southeast Asia. Their large-scale farming through repeated vegetative propagation and virtual mono-cropping has resulted in a variety of phyconomic issues. The efficacy of two European, commercial liquid extracts from the brown seaweeds, i.e., *Ascophyllum nodosum* (ANE), a fucoid, and *Laminaria digitata* (LDE), a kelp, on the direct formation of axes and survival of these two eucheumatoids was examined using tissue culture techniques. Seaweed apical sections (3–5 mm long) were cultured for 45 days, with different concentrations of ANE and LDE (i.e., 0.005, 0.05, 0.5, 1.5, and 2.5 mL L^{-1}) and compared with a control (UV-filtered seawater). Both liquid extracts improved the growth and morphogenesis of *E. denticulatum* and *K. alvarezii* in tissue culture. This study recommends a dilution rate of 0.05 mL LDE per L for *E. denticulatum* tissue culture resulting in a high percentage survival (i.e., 95.3 ± 0.9%), formation of direct axes (i.e., 95.3 ± 0.9%), and the longest length (i.e., 10.0 ± 0.3 mm) after a typical 45-day culture period. These data combined with the highest percentage formation of axes on day 14 (i.e., 88.0 ± 4.9%). However, 0.5 mL ANE per L is recommended for the tissue culture of *K. alvarezii*, given the relatively high final survival and direct axis formation (i.e., 96.2 ± 2.2%) and shoot length (i.e., 8.2 ± 0.1 mm). This study supports the application of these temperate, brown seaweed-derived extracts as phycobiostimulant enrichment in eucheumatoid micropropagation for mass production of plantlets for out-planting purposes.

Introduction

Eucheumatoids are red seaweeds belonging to the genera *Kappaphycus* and *Eucheuma*. They are economically important resources for the production of various types of carrageenan, which is a polysaccharide used in processed foods, cosmetics, and pharmaceutical applications (Bixler et al. 2011; Loureiro et al. 2017). Studies have also evaluated the potential of these red seaweeds as raw material resources for plant biostimulants and as feedstock for biorefinery and biofuel applications (Eswaran et al. 2005; Neish and Suryanarayan 2017; Meinita et al. 2012). The considerable global demand for these raw materials has led tropical and subtropical countries, particularly in Southeast Asia, to venture into the large-scale cultivation of these seaweeds (Hurtado et al. 2014). Almost 11.5 million MT wet weight of *Kappaphycus* and *Eucheuma* were produced in 2019 by Southeast Asian countries, sharing 98.88% of the global farmed seaweed production (Cai et al. 2021). Indonesia produced *c.* 8.1 million MT wet weight in 2020, compared to *c.* 1.5 million MT production of the Philippines (Rieve 2023). However, eucheumatoid-producing countries have been facing problems with dwindling /stagnating production attributed to pests and disease outbreaks, exacerbated by deteriorating farming environments and changing climate conditions (Msuya et al. 2022, Department of Agriculture Bureau of Fisheries and Aquatic Resources 2022). Conventional seaweed farming practices (e.g., repeated vegetative propagation of biomass and mono-cropping) resulted in negative phyconomic issues, including reduced strain vigor, lowered productivity, and increased susceptibility of cultivars to pathogens, diseases and pests (Hayashi et al. 2017; Hurtado et al. 2019).

Micropropagation by tissue culture via direct regeneration is one technique employed to produce higher yields of uniform species, in shorter periods, to increase the production of propagules for commercial nurseries, out-planting and farming (Reddy et al. 2008; Yokoya and Yoneshigue-Valentin 2011; Ali et al. 2020a; Hurtado et al. 2021). These practices generally involve the culture of axenic explants in a growth medium enriched with a range of macro- and micronutrients, vitamins, and plant growth regulators (Reddy et al. 2008). A number of studies have highlighted the potential applications of various types of seaweed extracts acting as phycobiostimulants and/or phycobioeffectors in seaweed micropropagation (the phyco prefix is used to differentiate seaweed-derived biostimulants from amino acids, humates and microbe-based biostimulants; Yunque et al. 2011; Jong et al. 2015; Tibubos et al. 2017; Ali et al. 2018a, 2020a; Souza et al. 2019; Batista de Vega et al. 2020; Umanzor et al. 2020, 2022). Commercially produced seaweed extracts come in liquid or soluble powder form, and many are commonly derived from brown seaweeds, e.g., *Ecklonia, Durvillaea, Laminaria* and *Ascophyllum*. Among the phycobiostimulants, *Ascophyllum* Marine Plant Extract Powder (AMPEP) from the North Atlantic fucoid *A. nodosum* (L.) Le Jolis is perhaps the most widely studied, and a number of publications support its efficacy when used on eucheumatoids (see Hurtado et al. 2021 for an extensive review). *Laminaria digitata* (Hudson) J.V. Lamouroux is another temperate brown seaweed; it is a kelp (order Laminariales) and is used as a biomass from which a commercial liquid extract is produced as a plant biostimulant. The extracts of *A. nodosum* and *L. digitata* are known to contain bioactive compounds such as high levels of betaines, organic osmolytic compounds, defense enzymes, and fatty acids that could trigger physiological responses and defense mechanisms in plants (Ertani et al. 2018; Hamed et al. 2018; Campobenedetto et al. 2021).

This study aimed to determine the efficacy of brown seaweed, liquid extracts from *A. nodosum* (ANE) and *L. digitata* (LDE) on the survival and direct formation (morphogenesis) of lateral axes of *E. denticulatum* and *K. alvarezii* using tissue culture techniques. This information provides valuable insights into improving culture practices by detailing the optimal treatments for mass production of propagules for nursery and out-planting.

Materials and methods

Preparation of Explants

Eucheuma denticulatum (*c.* 3–5 kg fresh weight) was collected from a seaweed farm site in San Dionioso, Iloilo, Philippines (11°17'17.9"N 123°06'09.9"E) on 22 June 2023. These seaweeds are cultivated by the fixed off-bottom line method (Hurtado et al. 2014). *Kappaphycus alvarezii* (*c.* 3 kg fresh weight) was obtained from the Southeast Asian Fisheries Development Center Aquaculture Department (SEAFDEC AQD), Tigbauan, Iloilo on 29 April 2023. The collected algae were immediately transported in styrofoam boxes to the Institute of Aquaculture Hatchery Complex, University of the Philippines Visayas, Miagao, Iloilo. Upon arrival, the boxes were left open for 30 minutes to allow for heat dissipation before the collected samples were transferred to 300 L fiberglass tanks. The seaweeds were acclimatized for one week prior to tissue culture experiments in fiberglass tanks under ambient conditions: 28 ± 2°C, 30 ± 3

psu, pH of 8 ± 0.2, and an irradiance of 130 ± 5 µmol photons m^{-2} s^{-1} (measured with an LI-250 light meter) with mild aeration.

Healthy and vigorous individuals from the algal stock were selected. Apical portions of the thalli were initially cut into 2–3 cm segments, surface-sterilized in 0.05% povidone iodine by brushing the surface using a soft artist's brush, rinsed in UV-filtered seawater, and finally cut into 2–3 mm thick cross sections using a sterile scalpel. The seaweed sections were then subjected to tissue culture experiments using two commercial brown seaweed liquid extracts from Algaia®, i.e., *Ascophyllum nodosum* (ALGANACT™ EVP10A; ANE) and *Laminaria digitata* (ALGANACT™ EVP6L; LDE). The characteristics of each liquid extract are shown in Table 1.

Table 1
Specifications of Algaia® brown seaweed liquid extracts from *Ascophyllum nodosum* (ANE) and *Laminaria digitata* (LDE)

	***A. nodosum* extract (ANE)**	***L. digitata* extract (LDE)**
Physicochemical Characteristics		
Color	Light brown	Very light brown
Odor	Sea spray	Sea spray
pH	3.2–4.2	3.2–4.2
Solubility in water	100%	100%
Density	1.05	1.05
Composition type		
Dry matter	> 10%	> 6%
Ash*	21–43%	31–53%
Mannitol*	9–19%	7–17%
Fucoidans*	2–5%	2–5%
Laminarins*	1–9%	1–9%
Uronic acids* (alginate units)	12–26%	16–30%
Phenolics*	4–10%	No data
Characteristics of Heavy Metals		
Arsenic	< 20 ppm	< 20 ppm
Lead	< 1 ppm	< 1 ppm
Cadmium	< 1 ppm	< 1 ppm
Mercury	< 1 ppm	< 1 ppm
*% on dry weight		

Tissue Culture Experiments

Two hundred sections each of *E. denticulatum* and *K. alvarezii* were maintained in 2 L culture vessels (1 section:10 mL), one containing ANE or LDE-seawater solution at different concentrations (i.e., 0.005, 0.05, 0.5, 1.5, 2.5 mL L^{-1}). As both extracts are acidic (i.e., pH of 3.5–4.5), the pH of the culture media was adjusted to 8 ± 0.2 using 1 N NaOH. A separate set of seaweed segments cultured in UV-filtered seawater

was prepared to serve as a control. Each treatment concentration and control had three replicates. All treatments were supplemented with 1 mL each of kinetin and IAA (plant growth regulators; Tibubos et al. 2017; Ali et al. 2018a).

The cultures were incubated in a walk-in culture room of the Seaweed Laboratory, UP Visayas under standard conditions of 23–24°C, incident irradiance of 110 ± 5 µmol photons m^{-2} s^{-1} provided by LED tubes, 13 L:11 D photoperiod, with moderate aeration (*c.* 2.0 L min^{-1}) for 45 days. Sampling and renewal of culture media were performed every seven days.

After the 45-d culture period, the survival and number of direct axes (lateral branches) formed by the thallus segments for each treatment and control were determined and calculated according to the following equations (Tibubos et al. 2017):

$$Survival\,(\%) = \left(\frac{final\,number\,of\,algal\,sections\,at\,Day\,45}{initial\,number\,of\,algal\,sections}\right)100 \quad \textbf{(Eq. 1)}$$

$$Direct\,axes\,formation\,(\%) = \left(\frac{number\,of\,sections\,with\,shoots}{initial\,number\,of\,algal\,sections}\right)100 \quad \text{(Eq. 2)}$$

Algal sections with shoots were also sampled for shoot length measurements using a Vernier caliper.

Statistical Analyses

Statistical analyses were performed using RStudio version 4.2.2 (R Development Core Team 2022). Data sets for multiple measurement comparisons were initially tested for normality using the Shapiro–Wilk test, followed by homogeneity of variances using Levene's method. One-way ANOVA and Tukey's HSD test were used to analyse differences among treatment concentrations by each seaweed extract (p = 0.05). For data sets where assumptions for normality and homogeneity were not satisfied, the non-parametric Kruskal–Wallis and Dunn's multiple comparison *posteriori* tests were used.

Results

a. Eucheuma denticulatum

Survival and morphogenetic direct axis formation of *E. denticulatum* micropropagules by the end of the 45-day culture period were positively influenced by both seaweed liquid extracts tested. The highest survival was observed at 0.005 mL ANE L^{-1} (97.7 ± 1.1%), although it was similar (p > 0.05) to those of 0.05 mL (95.5 ± 1.0%), 0.5 mL (94.8 ± 1.3%), and control (94.0 ± 3.0%; Fig. 1). A decreasing trend in survival was also observed with increasing ANE concentrations (i.e., a typical dose dependency). The lowest survival was observed at the higher concentrations of phycobiostimulant, i.e., 1.5 mL (51.8 ± 8.2%)

and 2.5 mL (50.3 ± 18.2%) LDE L^{-1}, indicating that testing dose response curves is very important to ascertain optimal ranges.

The percentages of direct axis morphogenesis were relatively similar among the ANE treatments and the control, ranging from 73.2 ± 13.8% (at 2.5 mL L^{-1}) to 97.5 ± 1.3% (at 0.005 mL L^{-1}; Fig. 2). Higher direct axis formation was observed with the lower LDE concentrations, with a maximum value of 95.3 ± 0.9% at 0.05 mL L^{-1}. While lateral shoots emerged as early as day 14 in all treatments, the highest direct axis formation on that day was observed in the 0.005 mL ANE (52.5 ± 18.1%) and 0.05 mL LDE (88.0 ± 4.9%) per L treatments. The morphogenesis of lateral axes in the control group on Day 14 was 40.7 ± 11.1% in the tissue culture experiment with ANE, and 60.8 ± 10.9% in that with LDE.

Among the ANE treatments, the longest direct axes were recorded at 0.005 mL L^{-1} (7.7 ± 0.2 mm) and 0.05 mL (7.9 ± 0.2 mm; Fig. 3). Among the LDE treatments, the longest direct axes were recorded in the 0.05 mL L^{-1} (10.0 ± 0.3 mm). The shortest direct axes were in the 1.5 mL (5.4 ± 0.1 mm) and 2.5 mL (5.2 ± 0.1 mm) L^{-1}.

b. Kappaphycus alvarezii

After 45 days of laboratory cultivation, algal sections of *K. alvarezii* treated with high LDE concentrations had the lowest survival rates (i.e., 32.9 ± 3.3% at 1.5 mL L^{-1} and 23.1 ± 2.7% at 2.5 mL L^{-1}; $p < 0.00004$; Fig. 4). The highest survival among the ANE treatments was observed at 0.5 mL L^{-1} (96.2 ± 2.2%), while the lowest was at 2.5 mL L^{-1} (45.5 ± 14.0%). However, there was no significant difference among ANE treatment concentrations and the control group.

The effects on axis morphogenesis were similar to the previously described survival rates of ANE- and LDE-treated *E. denticulatum* (Fig. 5). The 1.5 mL and 2.5 mL LDE L^{-1} treatment concentrations had the lowest percentage of shoot formation (18.0–24.9%) and were comparable with that of the control (29.9 ± 9.3%). Higher percentages of direct axis formation were observed in the ANE treatments, particularly at lower treatment concentrations (i.e., 87.8 ± 5.4% at 0.005 mL L^{-1}, 90.1 ± 4.8% at 0.05 mL L^{-1}, and 96.2 ± 2.2% at 0.5 mL L^{-1}). The first occurrence of lateral shoots was also observed on Day 14 in both liquid extracts. The highest direct axis formation on that day was in the 0.5 mL L^{-1} ANE (28.8 ± 12.0%) and 0.5 mL L^{-1} LDE (38.5 ± 15.6%) treatments, with no significant difference from their respective control groups owing to greater variability of measurements among replicates.

In terms of direct axis length, *K. alvarezii* treated with 0.5 mL (9.1 ± 0.3 mm), 1.5 mL (9.5 ± 0.2 mm), and 2.5 mL (8.6 ± 0.4 mm) LDE per L were the longest (Fig. 6). For the ANE treatments, the longest direct axes were recorded at the 0.5 mL L^{-1} (8.2 ± 0.1 mm).

Discussion

Various environmental issues and biophysical stresses threaten the eucheumatoid industry, such as reduced vigor and productivity of the most commonly farmed seaweed cultivars, increased frequency of diseases and pests, and intensifying climate-driven tropical cyclones (Hurtado et al. 2019; Ward et al. 2020; United Nations Environment Programme 2023). Selected phycobiostimulants which are normally utilized for land plants have been applied in seaweed micropropagation to produce superior quality propagules for commercial cultivation (Hurtado et al. 2021). A number of commercial seaweed extracts used in agricultural and horticultural industries are already available on the market (Khan et al. 2009; Shukla et al. 2019); they are derived mostly from brown seaweeds such as *A. nodosum*, *L. digitata, Macrocystis pyrifera* (L.) C. Agardh, *Ecklonia maxima* (Osbeck) Papenfuss, and *Durvillaea antarctica* (Chamisso) Hariot.

The present study revealed the efficacies of selected European brown seaweed liquid extracts from *A. nodosum* (ANE) and *L. digitata* (LDE) in the tissue culture of *E. denticulatum* and *K. alvarezii*. Both eucheumatoid species responded differently in terms of their survival, direct axis formation and length at varying concentrations of ANE and LDE. The use of 0.05 mL LDE L^{-1} resulted in high survival, direct axis formation and the longest shoot length of *E. denticulatum* in tissue culture. *K. alvarezii* had fairly high growth and survival in the ANE treatments; among them, the 0.5 mL L^{-1} concentration yielded the longest shoots. Although the higher LDE treatment concentrations (i.e., 0.5, 1.5, and 2.5 mL L^{-1}) in *K. alvarezii* were recorded to have the longest axis length, they also had the lowest survival and number of direct axes. Thus, such interpretation of results and recommendation as to which phycobiostimulant and concentration is best in tissue culture of *K. alvarezii* should be done with caution. The purpose of tissue culture is to produce higher yields of vegetative propagules for nursery and large-scale cultivation of eucheumatoid seaweeds (Hurtado et al. 2009; Ali et al. 2020a). In other studies, 3 mg L^{-1} of AMPEP powder extract only, or supplemented with plant growth regulators (3 mg L^{-1} AMPEP + PGR) was found to be the optimal concentration for the induction of direct shoots of *K. alvarezii* and *K. striatus* (Hurtado et al. 2009; Ali et al. 2018, 2020a), while lower concentrations of AMPEP + PGR were reported to be optimal by Yunque et al. (2011). It is, however, difficult to compare such results of previous studies to the present study, considering the differences in soluble solids, bioactive constituents, and types of extraction processes used for different commercial seaweed extracts. While phycobiostimulants contain bioactive compounds that are inherent in seaweeds, new compounds may be formed by hydrolytic processes during the process of extraction (Vaghela et al. 2023). Moreover, the present experiment chose to have a persistence of liquid seaweed extracts at low doses in the seawater medium throughout the 45-day culture period, rather than dipping the cultivars at higher concentrations of phycobiostimulant for a time ranging from 30–60 min (Borlongan et al., 2011; Hurtado et al. 2012; Ali et al. 2018b, b). The resulting color characteristics of the culture media in the present study also affected light penetration and perhaps perceived wavelength, subsequently reducing photosynthesis and growth rate (Reddy et al. 2003; Tirtawijaya et al. 2022); LDE a resulted in a cloudy cream coloration in higher concentrations, while ANE exhibited a light brown resulting in reduced light penetration into the culture medium (i.e., 41–50 µmol photons m^{-2} s^{-1}) and hence light limitation (Supplementary Fig. 1). A study by Yong et al. (2014)

revealed that the optimum irradiance for direct regeneration of *K. alvarezii* in a culture vessel was 75 µmol photons m^{-2} s^{-1}.

Meristematic cell growth in the seaweed sections gave rise to filamentous outgrowths from both the medullary and cortical regions of the cut surface, which eventually aggregated and consequently led to the formation of shoot primordia (Hurtado et al. 2009). The potential of ANE and LDE to induce the morphogenesis of direct axes in the micropropagation of *E. denticulatum* and *K. alvarezii* can perhaps be attributed to the composition of bioactives such as mannitol, fucoidan, and laminarin. These storage carbohydrates and their derivatives function as osmoregulators or osmoprotectants; they are known to be elicitors of defense responses and scavengers of reactive oxygen species in higher plants, thereby mitigating the damaging effects of environmental stress (Iwamoto and Shiraiwa 2005; Dittami et al. 2011; Battacharyya et al. 2015; Rioux and Turgeon 2015). Additionally, phytohormones and plant growth regulators such as auxin, cytokinin, and gibberellins have been reported in some brown seaweed extracts (Tarakhosvskaya et al. 2007; Sharma et al. 2014; Vaghela et al. 2023), which are useful as biostimulants in agriculture and horticulture (Khan et al. 2009; Reddy et al. 2017). The assumed presence of these plant growth-promoting substances or their precursors in the seaweed liquid extracts, even though in trace amounts, in the present study may have stimulated meristematic growth and the formation of direct axes, thereby improving the success rates for micropropagation.

In principle, biostimulants (e.g., humic acids and seaweed extracts) are substances that can promote plant growth and stress relief when applied in minute quantities, contrary to commercial fertilizers and soil enhancers that are applied in larger amounts (du Jardin 2015; Sible et al. 2021). Further investigation of the nutrient availability, plant growth regulators, and bioactive metabolites in the *A. nodosum* and *L. digitata* liquid extracts is integral to better elucidate their effects on the growth and direct regeneration of explants. The microbiological composition of the seaweed extracts may also need to be explored for the potential associations between the microbial communities in the extracts and the microbiome of the cultured seaweeds, including novel metabolic products derived from such interactions (Vaghela et al. 2023), which could have a role in improving their growth and tolerances toward abiotic and biotic stresses. Similar to the pathobiome concept (Bass et al. 2019), interactions between the microbiota in the seaweed liquid extracts, the host (i.e., seaweed explants), and environment, or any quorum sensing impacts therein, could positively or negatively influence the risk of disease in the micropropagated strains in sea-based nursery and field cultivation by shaping their microbiome and immune response.

Conclusion

The present study confirms the biostimulant effectiveness of the brown seaweed phycobiostimulants from *A. nodosum* (ANE) and *L. digitata* (LDE) in the tissue culture of *E. denticulatum* and *K. alvarezii*. Results of dose-response relationships suggest the use of 0.05 mL LDE L^{-1} for *E. denticulatum* and 0.5 mL ANE L^{-1} for *K. alvarezii* to achieve maximum growth and morphogenesis of microplantlets. The application of such seaweed-derived liquid extracts can be a potential alternative to existing nutrient or

biostimulant enrichment techniques in tissue culture for large-scale production of good quality and high-yielding eucheumatoid cultivars.

Declarations

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Authors' contribution Conceptualization: AQH, ATC & IAB; Formal analysis and investigation: HCG, ALD & IAB; Writing – original draft preparation: IAB & ALD; Writing – review and editing: IAB, AQH, ATC & FH; Resources: FH, ATC, AQH & IAB; Funding acquisition: IAB

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Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interest The authors declare no conflict of interest regarding the publication of this paper.

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Figures

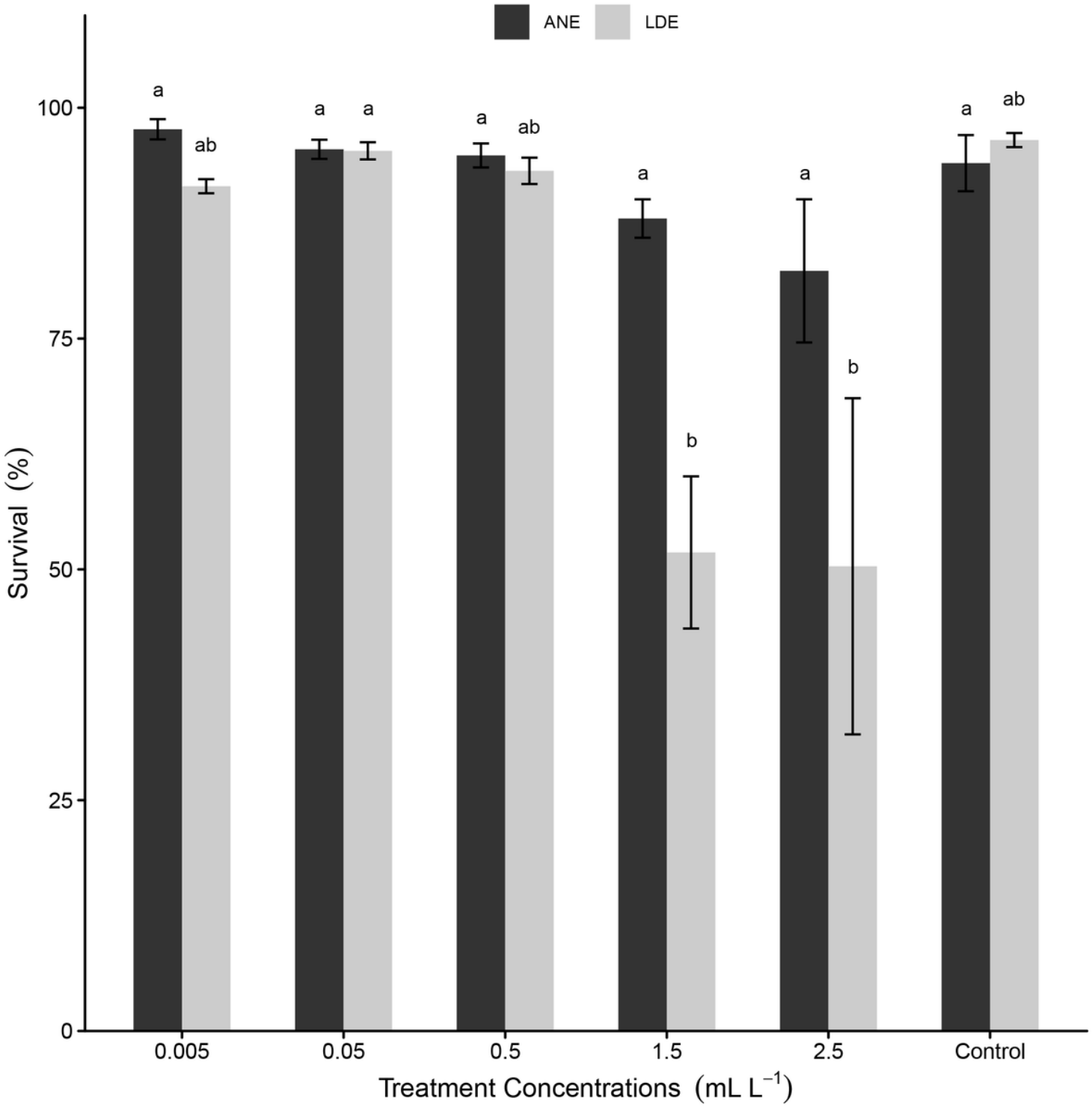

Figure 1

Survival (mean percentage ± SEM, *n* = 3) in *Eucheuma denticulatum* after 45 d of culture at varying concentrations of seaweed liquid extracts (ANE, *Ascophyllum nodosum* extract; LDE, *Laminaria digitata* extract). Means with different letter(s) were significantly different at $p < 0.05$.

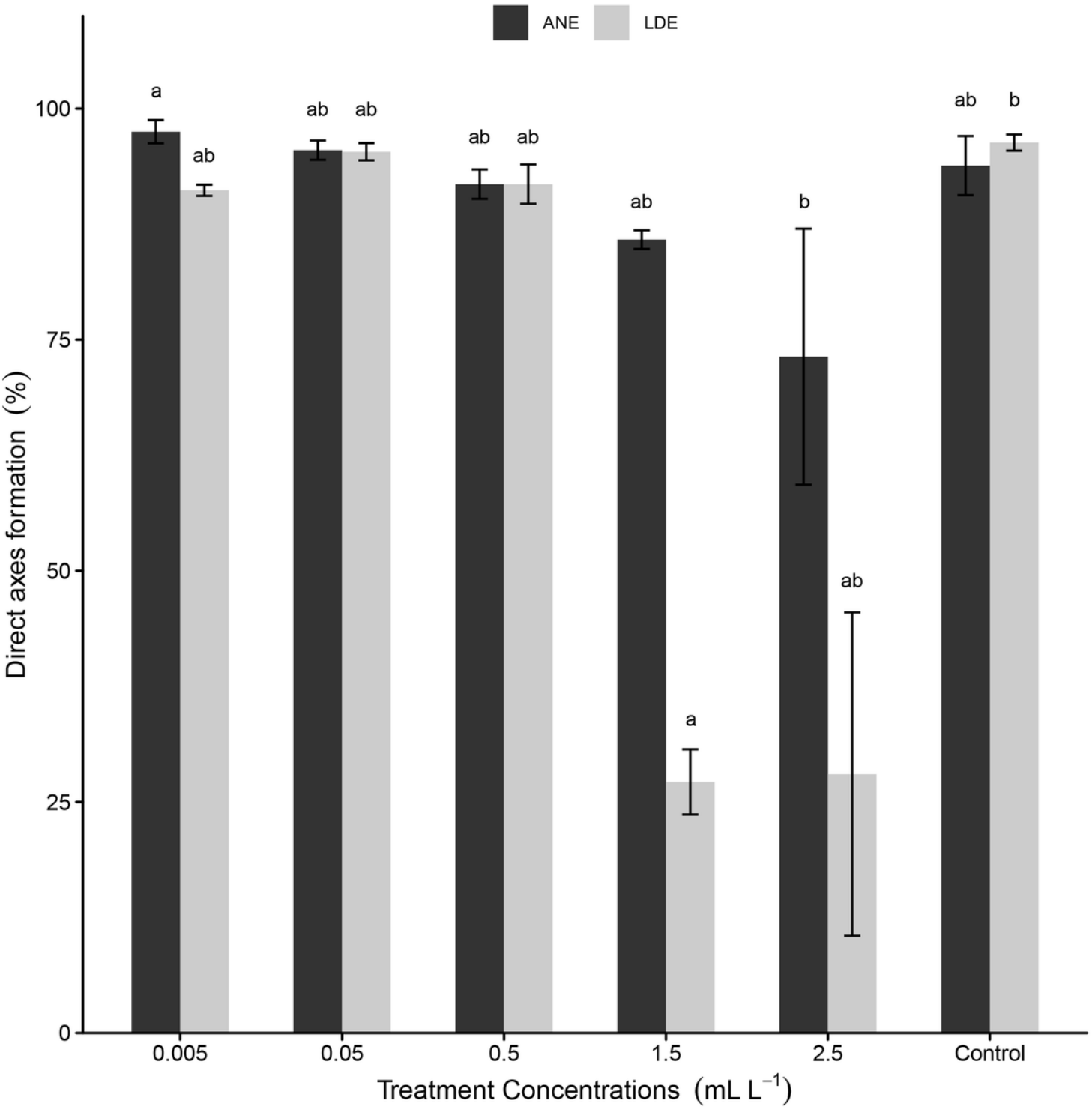

Figure 2

Direct axis formation (mean percentage ± SEM, *n* = 3) in *Eucheuma denticulatum* after 45 d of culture at varying concentrations of seaweed liquid extracts (ANE, *Ascophyllum nodosum* extract; LDE, *Laminaria digitata* extract). Means with different letter(s) were significantly different at $p < 0.05$.

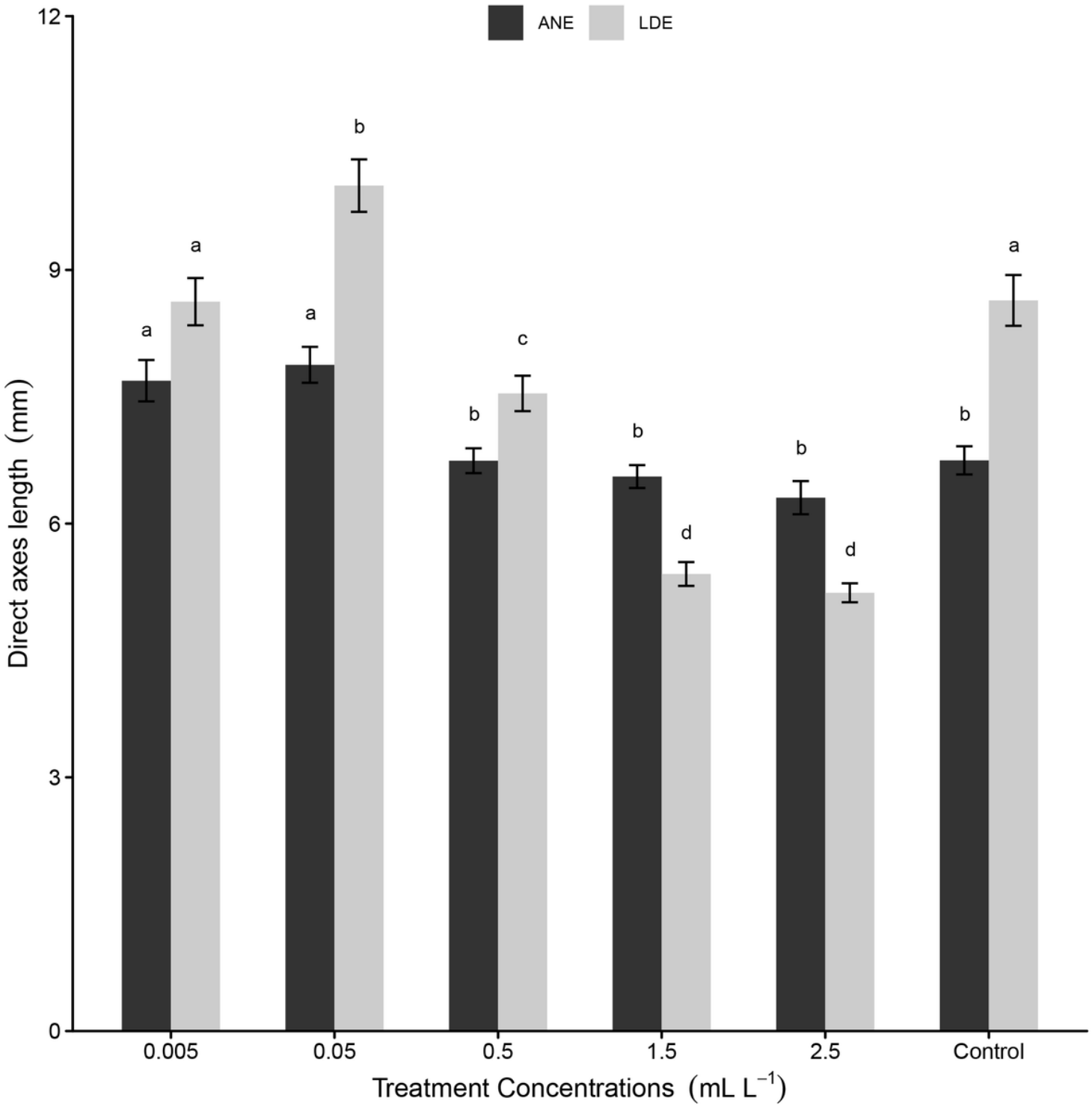

Figure 3

Direct axis length (mm; mean ± SEM) in *Eucheuma denticulatum* after 45 d of culture at varying concentrations of seaweed liquid extracts (ANE, *Ascophyllum nodosum* extract; LDE, *Laminaria digitata* extract). Means with different letter(s) were significantly different at $p < 0.05$.

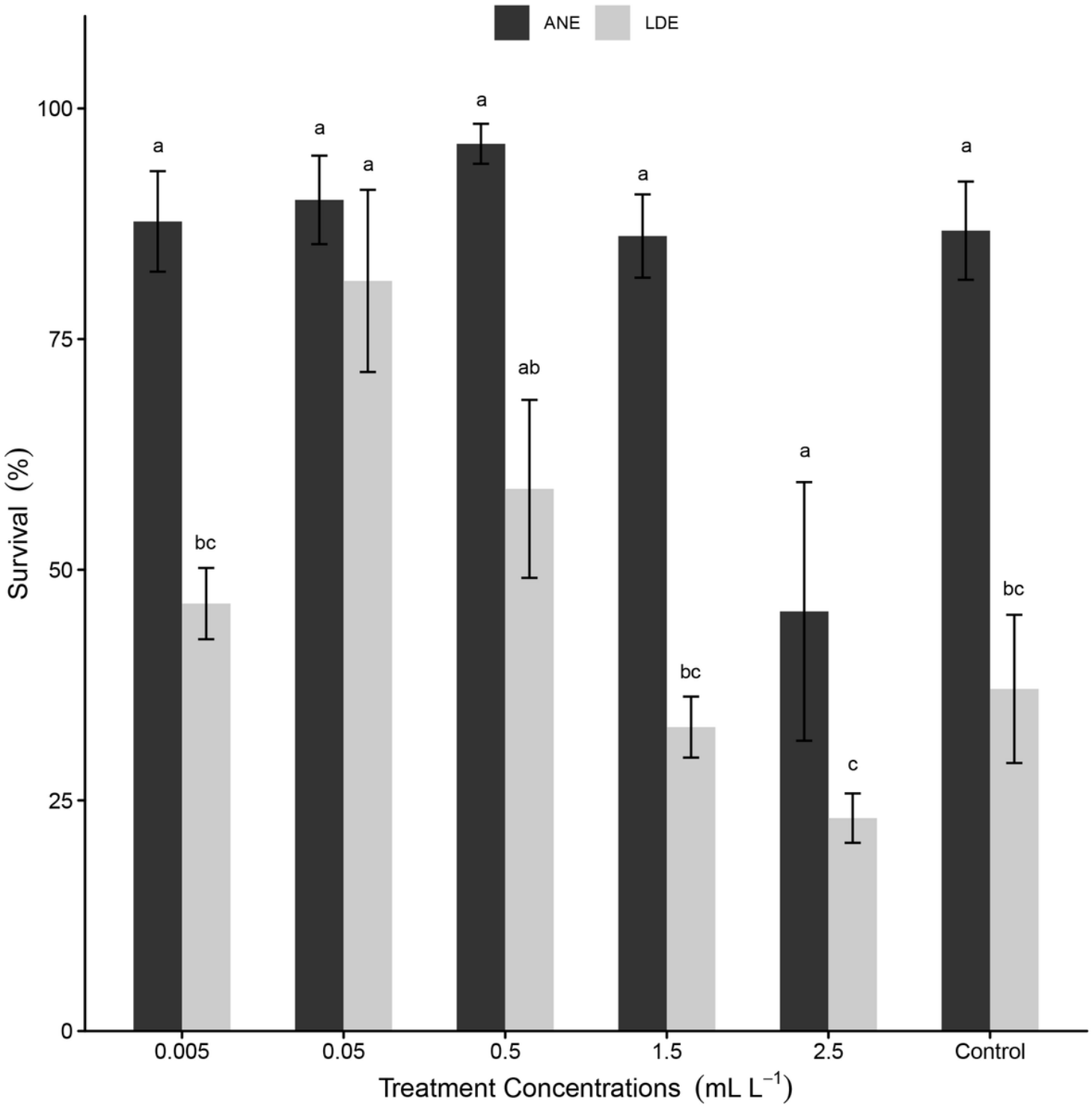

Figure 4

Survival (mean percentage ± SEM, *n* = 3) in *Kappaphycus alvarezii* after 45 d of culture at varying concentrations of seaweed liquid extracts (ANE, *Ascophyllum nodosum* extract; LDE, *Laminaria digitata* extract). Means with different letter(s) were significantly different at $p < 0.05$.

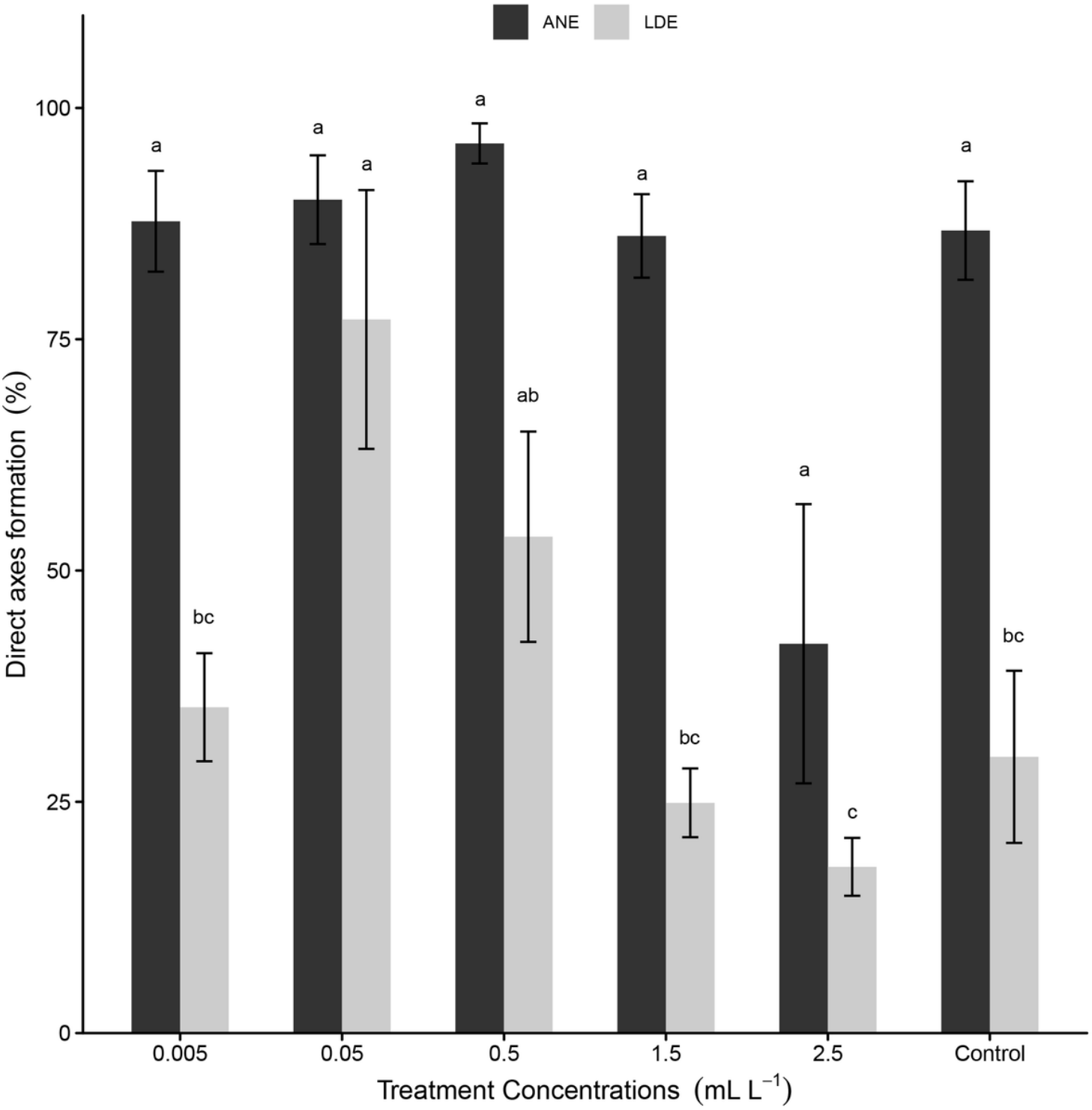

Figure 5

Direct axis formation (mean percentage ± SEM, *n*= 3) in *Kappaphycus alvarezii* after 45 d of culture at varying concentrations of seaweed liquid extracts (ANE, *Ascophyllum nodosum* extract; LDE, *Laminaria digitata* extract). Means with different letter(s) were significantly different at $p < 0.05$.

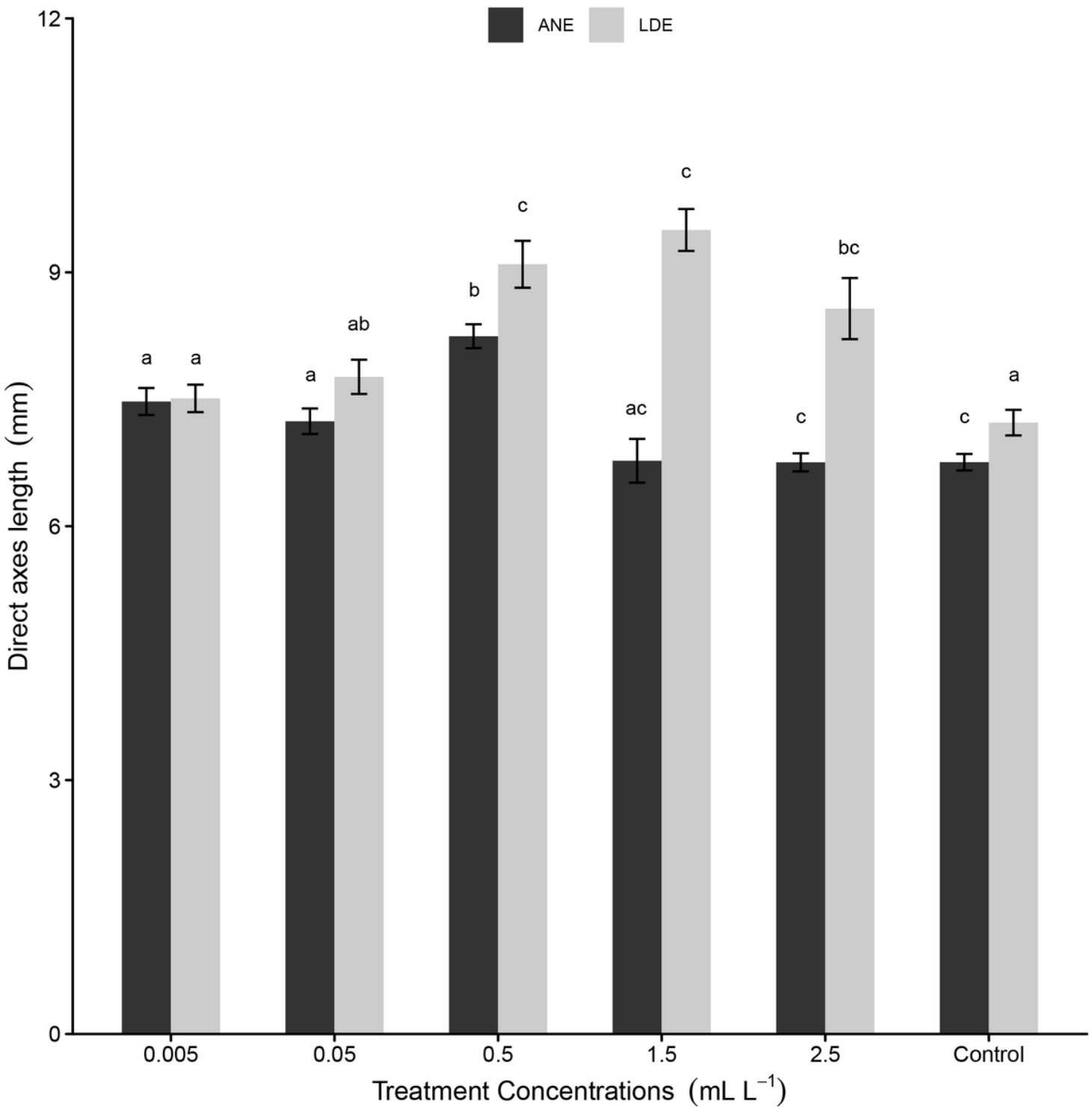

Figure 6

Direct axis length (mean ± SEM) in *Kappaphycus alvarezii* after 45 d of culture at varying concentrations of seaweed liquid extracts (ANE, *Ascophyllum nodosum* extract; LDE, *Laminaria digitata* extract). Means with different letter(s) were significantly different at $p < 0.05$.

Supplementary Files

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- SupplementaryFig.1LDEANEcultures.pdf