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EFFECT OF DIETARY SUPPLEMENTATION WITH *Lactiplantibacillus plantarum* I ON QUEEN SCALLOP *Aequipecten opercularis* UNDER SIMULATED CLIMATE CHANGE CONDITIONS

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ABSTRACT

This study examined the effects of dietary supplementation of queen scallop *Aequipecten opercularis* with an indigenous strain of lactic acid bacteria (LAB), *Lactiplantibacillus plantarum* I, previously isolated from its digestive tract, on gut microbial populations and growth rates during cultivation under simulated climate change conditions (pH 7.8, T = 16 ± 2 °C). After one month of feeding, the results showed a noticeable reduction in aquaculture diseases causing pathogens while maintaining sufficient viable *Lpb. plantarum* I cells. A higher pH and temperature resulted in higher growth rates, measured by the weight and length of scallops, compared to the control group. The results obtained shed light on the influence of the addition of lactic acid bacteria on the growth of bivalve mollusks under normal and climate change conditions, and provide control of pathogenic microorganisms. In the context of climate change, host-pathogen interactions need to be recognized and put under control by applying natural solutions to minimize the environmental footprint.

How to Cite

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INTRODUCTION

A pressing global challenge revolves around ensuring an adequate food supply, with a particular focus on protein sources, to meet the needs of the expanding human population. This challenge is exacerbated by the current situation where many wild food stocks are either fully depleted or overexploited (Houghton et al., 2001). Shellfish have garnered significant attention as an essential component of a well-rounded, healthy diet due to their rich nutritional content (Laing, 2022; Kovačić et al., 2023). European shellfish aquaculture is mainly based (94%) on oysters and mussels, clams (5%), while cockles, ark shells, and scallops (1%) are responsible for only a small European market share (FAO, 2020). In Croatia, shellfish aquaculture is based mainly on the Mediterranean mussel *Mytilus galloprovincialis* and the European flat oyster *Ostrea edulis* (MA, 2021). The lack of diversification in shellfish culture may result in negative effects due to stochastic events, e.g. new pathology outbreaks, strong sea storms or climatic changes (El Bassi et al., 2009). Interest in scallops from the *Pectiniidae* family for human consumption has increased significantly in recent years (Villamil et al., 2010; Kamermans and Saurel, 2022). Among them, queen scallop *Aequipecten opercularis* (Linnaeus, 1758) is distributed along the Mediterranean Sea and may offer interesting prospects for aquaculture. The queen scallop market value is currently comparable to that of the depleted wild king scallop *Pecten jacobeus* (Linnaeus, 1758) and its use in aquaculture might contribute to a reduction in the negative impacts of fishing gear on the sea bottom (Caldeira and Wickett, 2003; Doney et al., 2009; Muñoz-Atienza et al., 2014).

Climate change and ocean acidification (CC-OA), caused by mankind's emissions of carbon dioxide and other greenhouse gasses, represent a major threat today and in the future (Karvonen et al., 2010; Antolović and Antolović, 2012). According to the predictions by Garcia-Soto et al. (2020), the expected temperature increase for the next 100 years is around 2 °C, while the expected pH decrease is 0.2 units. Climate change can impact aquaculture through a variety of mechanisms varying by location and type with implications for future productivity (Marčeta et al., 2016). Shellfish aquaculture represents a significant area affected by shoreline erosion or coastal floods, including the expansion of pathogens from wastewater related to climate change (Hughes et al., 2012). The increase in seawater temperature can induce pathogen development causing disease transmission and host vulnerability, although a subset of pathogens might decrease with warming ultimately not causing disease (Duncan et al., 2016). Climate change and ocean acidification will have direct consequences on shellfish aquaculture since bivalves have been identified as a particularly vulnerable group affected by the combined effects of CC-OA (Bratoš et al., 2004). Since vulnerabilities of aquaculture to CC-OA are currently poorly understood, research based on the prevention and sustainability of shellfish farming under these conditions is more than necessary given that climate change also causes indirect consequences for the marine environment in the form of changing patterns of salinity, frequency of extreme weather conditions and algal blooms, spread of invasive species and diseases (Alleway et al., 2019).

Marine species have dealt with CC-OA throughout their evolutionary history, however, the fast climate changes that are occurring may affect the capacity of organisms to adapt to these variations, affecting their survival, physiology and growth (Stavrakidis-Zachou et al., 2021; Esposito et al., 2022). Temperature and pH are two of the most important physical factors affecting intertidal organisms such as shellfish (Kovačić et al., 2023). While temperature is considered to affect energy balance and level of activity (Domínguez et al., 2021), pH influences the physiological processes of marine shellfish (Carregosa et al., 2014; Zittier et al., 2015). When exposed to CC conditions, such as severe variations in temperature and ocean acidification, shellfish usually close their valves which reduces feed intake (Carregosa et al., 2014), and show slower growth rates, and consequently alterations in commercial quality (Bertolini et al., 2021).

CC-OA will promote the growth and distribution of pathogenic microorganisms (e.g. viruses, bacteria, protozoans) (Zgouridou et al., 2022), impacting the feeding behavior of finfish and shellfish (Khan et al., 2020; Castello et al., 2023). The most common shellfish bacterial infections are caused by V*ibrio, Salmonella, Aeromonas* and *Listeria monocytogenes*. Abuse of antibiotics in aquaculture can cause their accumulation in humans and the emergence of antibiotic-resistant bacteria (Venugopal et al., 2017). Therefore, it is necessary to find alternative natural antimicrobial compounds or functional feed components (Kesarcodi-Watson et al., 2008). Lately, the emphasis has been put on the use of lactic acid bacteria (LAB). It has been reported that a variety of indigenous LAB provided higher survival rates, improved gut microbiome, mucosal simulation, as well as immune response and antioxidant properties of fish and shellfish (Villamil et al., 2014; Ringø et al., 2019). The application of such bacteria can increase the availability of nutrients and energy to the host while simultaneously inhibiting pathogen growth (Ferreira et al., 2021). LAB isolated from marine species are preferable food supplementation for fish and shellfish since they are already acclimatized to the marine environment (Kovačić et al., 2017).

Given all the above, the aim of this study was to investigate the addition of marine LAB *Lactiplantibacillus plantarum* I as a food supplement to understand how it affects the microbial population of queen scallop under CC conditions. Previous aquaculture studies concerning queen scallop have been conducted by Kovačić et al. (2023), and biochemical properties showed potential for

the human diet. For reasons such as global warming and pollution, scallops can be infected by pathogens. This leads scientists to search for alternative natural antimicrobial compounds such as LAB for improving scallop health status and better growth rates. Thus, the growth rates of scallops in captivity under CC conditions and LAB cultures were monitored for the first time.

MATERIALS AND METHODS

Samples

Live queen scallops *Aequipecten opercularis* (Linnaeus, 1758) were collected with a fishing vessel bottom-trawling net at a depth of 49 meters, 2 nautical miles south-east off the Albanež shoal, Istria, Croatia, within the E2 fishing zone (44°43'58.49"N, 13°56'48.94"E) in October 2020. The collected specimens (N = 160) were immediately transported to the Aquarium Pula and placed in a flow-through 1900 L tank for one week of acclimatization. The water flow was kept at a rate of 200 L per hour. The tanks were cleaned by siphoning the bottom of the tanks each day. The seawater in the tank was obtained from a seawater bore located near the premises of Pula Aquarium and therefore this water was used in experimental tanks.

Strain

Lactiplantibacillus plantarum I was isolated from the digestive tract of queen scallop, identified with MALDI-TOF, and characterized in our previous research (Čanak et al., 2023). The culture was stored in a 50 % glycerol solution (Gram-Mol, Zagreb, Croatia) at –80 °C. Before experiments, the strain was recovered in MRS broth (Biolife, Milan, Italy) and incubated without shaking at 37 °C.

Survival of Lpb. plantarum I strain in conditions of ecological valence limit

Survival was conducted in an Erlenmeyer flask with autoclaved seawater (121 °C, 15 min), pH 7.8, at a temperature of 16 ± 2 °C. The pH was adjusted after autoclaving with sterile acid or alkali. The experiment was conducted with an initial number of 10^{10} CFU/mL. Aliquots of 1 mL were taken after 7, 14, 21 and 28 days to determine the number of viable cells. From samples containing bacterial cells, decimal dilutions were prepared in sterile water and spread on MRS agar plates (Biolife). The plates were incubated at 37 °C for 24 h. Number of cells was expressed as CFU/mL.

Preparation of wet biomass for supplementation and feeding

After overnight growth of *Lpb. plantarum* I in MRS broth (Biolife) at 37 °C, the bacterial cells were aseptically harvested by centrifugation (6000 rpm/10 min), washed twice with physiological saline, and resuspended in sterile physiological saline. For the total viable count (TVC) the standard dilution method was used on MRS agar (Biolife) after incubation at 37 °C for 24-48 h. The final number of 10^9 viable bacterial cells of *L. plantarum* I per g of wet biomass was obtained.

Cultivation of scallops in conditions of ecological valence limit

Following the one-weeklong acclimatization period, three sets of 30 individuals were put in 190 L closed-system tanks with a working volume of 160 L, aerated with single aeration stones for 30 days. The seawater in the tanks was constantly filtered with a mechanical aquarium filter, except at the time of feeding (9 AM-3 PM). Every other day 20% of the seawater was changed. The photoperiod was kept at 12 hours of light and 12 hours of darkness.
The scallops were fed daily to satiation with 100 ml live algae culture mix of *Tetraselmis* sp. (5×10^5 cell/mL), *Nannochloropsis* sp. (30×10^5 cells/mL) and *Phaeodactylum* sp. (12×10^5 cells/mL), which resulted in about 517×10^4 cells available per individual. Any excess food was removed by mechanical filtration after the feeding period was over. Algae concentration was calibrated in relation to the number of scallops inhabiting the tank, i.e. food quantity was reduced as the number of individuals was reduced. In the experimental tank, scallops received daily supplementation of LAB culture, maintained in circulation via an aeration stone. Furthermore, the algae concentration was adapted based on the scallop population in the tank and the addition of LAB culture. During the feeding period, the water flow was restricted.
The scallops in the control tank were kept under natural seawater conditions without fluctuations in temperature and pH, and were fed only with algae culture. In the second experimental tank, the scallops were exposed to climate change conditions: elevated temperature of approximately 2 °C and reduced pH of 0.2 (ΔpHΔT). In the third tank, the scallops were exposed to LAB culture (~10^2 CFU/ mL of the tank) in parallel running under climate change conditions (ΔpHΔT + LAB). The climate change conditions were simulated according to the predictions by Salgado-García et al. (2020). Temperature, pH, and dissolved oxygen were measured daily with the Hanna HI98193 multiparameter probe. During the experiment, the average temperature in the control tank was 15.65 ± 0.50 °C, pH was 7.88 ± 0.07 and dissolved oxygen (DO) was 92%, while in the experimental tanks, the temperature was 17.00 ± 0.49 °C, pH was 7.64 ± 0.08 and DO was 92% (Figure 1). LAB culture was added every day during feeding only in the third tank where the temperature was elevated and pH lowered, as mentioned before. During feeding, the water flow was kept closed. Excess food and faeces were removed by siphoning the bottom of the aquarium.

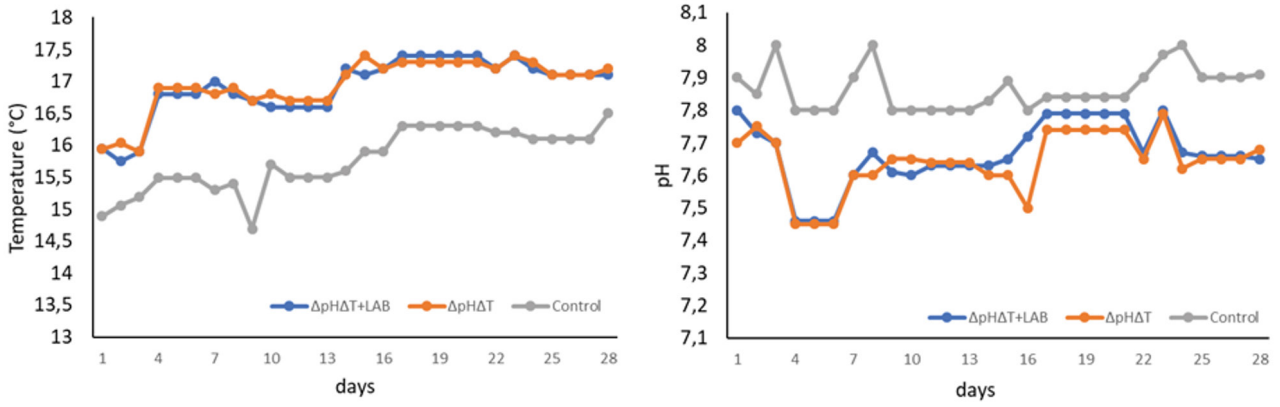

Fig 1. Temperature and pH levels in the control tank (Control), tank under climate change conditions (ΔpHΔT), and tank under climate change conditions and addition of LAB cultures (ΔpHΔT + LAB)

Microbiological analysis

At the end of the experiment, microbiological analysis was performed on 10 randomly selected scallops from each tank. These specimens were put on ice in a damp cloth and sent in a refrigerated cool box to the Laboratory for General Microbiology and Food Microbiology, Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia for the microbiological analysis. Total aerobic mesophilic bacteria were counted after incubation on nutrient agar (Merck, Darmstadt, Germany) at 37 °C for 48 h (HRN ISO 4833). *Enterobacteriaceae* were counted after incubation on Violet Red Bile Glucose (VRBG) agar (Biolife) at 37 °C for 48 h (ISO 21528-1:2017). Coagulase-positive staphylococci were counted after incubation on Baird-Parker (BP) agar (Biolife) at 37 °C for 24 h (ISO 6888-1:2021) and sulphite-reducing clostridia were counted using sulphite iron agar (Merck) at 37 °C for 72 h (ISO/CD 15213-2). Bacteria of the *Vibrio* genus were detected using TCBS Kobayashi agar (Biolife) at 37 °C for 48 h (ISO 21872-1:2017). The number of LAB was determined using MRS agar (Biolife) incubated at 37 °C for 48 h. The microbial growth was determined using traditional plate counting and the results were expressed as colony-forming units per gram of shellfish meat (CFU/g).

Scallop growth rate

For each individual of *Aequipecten opercularis*, total weight and length were recorded at the beginning and the end of the experiment to calculate growth and specific growth rates. The total length (LT: maximum length along the anterior-posterior axis) was measured using a stainless steel caliper (0.1 mm), and the weight was measured with a digital balance to the nearest 0.1 g (TW: total weight). The growth rate (GR) and specific growth rate (SGR) of shell length and wet weight were calculated according to the following equations:

$$\textit{Growth Rate length } (GR_l) = (L_f - L_i) / t$$

$$\textit{Growth Rate weight } (GR_w) = (W_f - W_i) / t$$

$$\textit{Specific Growth Rate length } (SGR_l) = n\,(\ln (L_f) - \ln (L_i)) / t * 100$$

$$SGR_w = \ln (\ln (W_f) - \ln (W_i)) / t * 100$$

where

L_f = final average shell length,
W_f = wet weight at the end of the experiment,
L_i = initial average shell length,
W_i = wet weight at the beginning of the experiment,
ln = natural logarithm and
t = experimental time in days.

Statistical analysis

All measurements of microbiological analyses were carried out in triplicates. The results are expressed as mean value ± standard deviation (S.D.). SigmaPlot 13.0 scientific data analysis and graphing software was used. The growth rate data were subjected to one-way ANOVA to test the difference between control and experimental tanks, and when significant differences were found, the means were subjected to *post hoc* analysis using Tukey's HSD test ($P < 0.05$). The normality of distributions and homogeneity of variances were assessed using the Kolmogorov–Smirnov test and Levene's test, respectively. All statistics were analysed by Statistica 9.0 (StatSoft).

RESULTS AND DISCUSSION

To manifest their positive protective properties, LAB must be present in higher numbers, thus it was important to test the strain survival under simulated climate change conditions before starting an experiment with shellfish. A high survival rate was noticed for *Lpb. plantarum* I, showing a good number of viable cells even after one month (10^7 CFU/mL) in comparison to the initial number of 10^{10} CFU/mL.

These results correlate positively with previous studies in which LAB from marine organisms showed a good survival rate in seawater (De Silva and Soto, 2009; Robson et al., 2016; Čanak, 2020). However, it needs to be emphasized that this is the first study to investigate the survival of LAB under climate change conditions and there are no comparable studies to date.

In view of the above results and since *Lpb. plantarum* I showed the best characterization results in our previous study, this strain was used as a nutrition supplement during the feeding of queen scallop in terms of ecological valence. After one month of supplemented feeding in the tank under simulated conditions of ecological valence (ΔpHΔT), there was already a decrease in the number of all tested microorganisms compared to the control sample, while in the tank with added LAB (ΔpHΔT + LAB) this number was further reduced. The most significant decrease was recorded in the number of aerobic mesophilic bacteria present in the control tank at a concentration of 2.6×10^8 CFU/g by setting the environmental valence conditions. The concentration was reduced to 5×10^5 CFU/g, while the number of living cells was further reduced to 1.2×10^4 CFU/g by adding LAB to the tank. A similar situation was observed for the concentration of coagulase-positive staphylococci, which was 1.5×10^4 CFU/g in the control tank. In the tank under simulated climate conditions, it changed to 5.1×10^3 CFU/g, and with the addition of LAB + climate change conditions it dropped to 8×10^2 CFU/g. These results agree with previous studies where marine LAB showed good inhibitory activity toward pathogens (Čanak et al., 2018; Ferreira et al., 2021). Since we excluded the inhibitory effect of lactic acid in our previous research, good growth inhibition of pathogenic microorganisms could be a consequence of antimicrobial compounds, including diacetyl, hydrogen peroxide, or bacteriocin presence, which must be further examined. Sulphite-reducing clostridia were not detected in any of the tested tanks as well as *Enterobacteriaceae*, indicating good hygienic conditions. Bacteria of the *Vibrio* genus were not detected in any of the samples, which was expected since this bacterium has a low incidence in the winter months when the sea is colder (Čanak et al., 2018). The scallops in the control tank reached the weight of the highest specific growth rate (SGRw) (0.24%) after 30 days of feeding, followed by the growth rate weight (GRw) (0.03 mm day^{-1}). The specific growth rate length (SGRl) and growth rate length (GRl) of scallops reached 0.01 % and 0.007 mm day^{-1}, respectively (Figure 2). The changes in the ΔpHΔT tank affected positively the SGRw (0.36 %), SGRl (0.06 mm day^{-1}), GRw (0.006 %) and GRl (0.03 mm day^{-1}) of scallops, when compared to the control tank. The SGRw of scallops in the control tank was significantly higher (Table 1) in comparison to the ΔpHΔT tank (Tukey HSD test, $P < 0.01$) and lower for scallops in the ΔpHΔT + LAB tank (Tukey HSD test, $P < 0.05$).

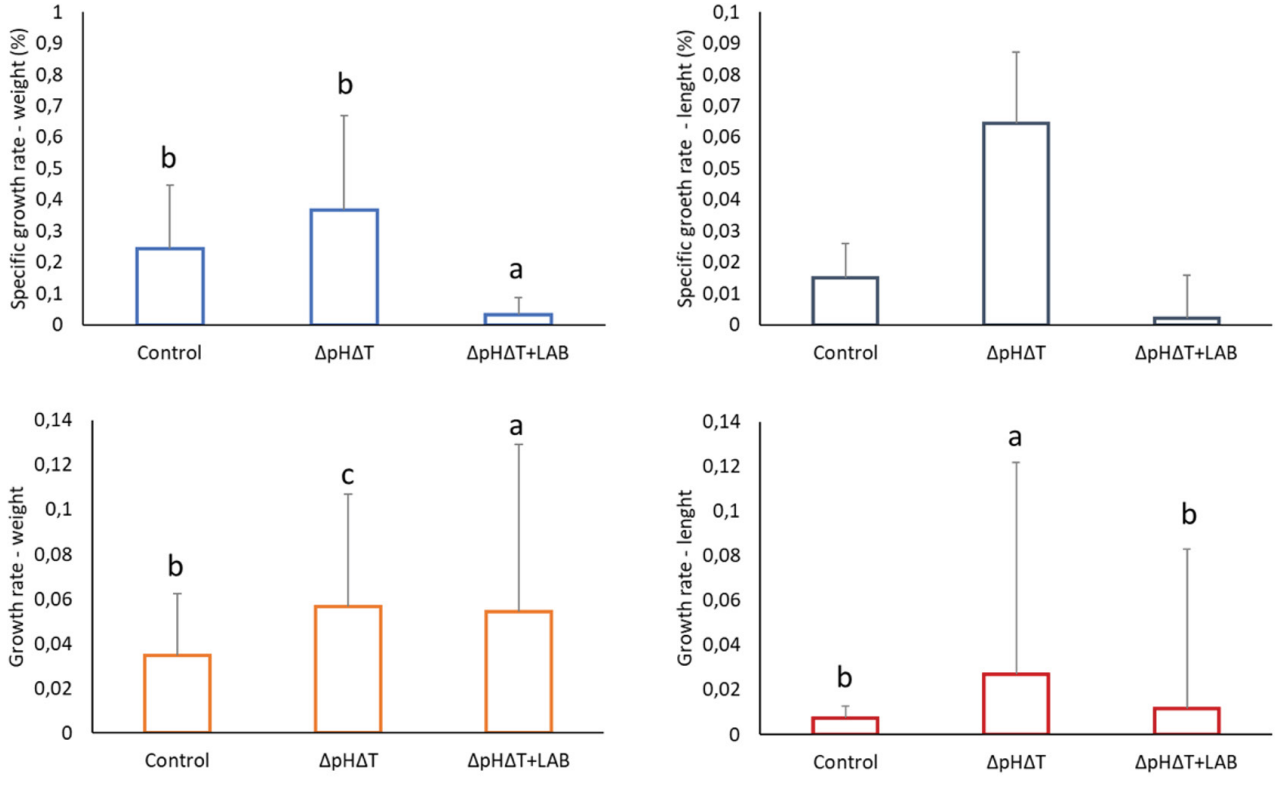

Fig 2. Specific growth rates (%) and growth rates (mm day^{-1}) based on weight and length of *A. opercularis* exposed to climate change conditions (ΔpHΔT) and climate change conditions with the addition of LAB cultures (ΔpHΔT + LAB) during 4 weeks. The means labelled by different letters are significantly different (Tukey's HSD test, $P < 0.05$)

Table 1. Results of one-way ANOVA testing differences in growth rate weight (GR_w) and length (GR_l), and specific growth rate weight (SGR_w) and length (SGR_l) between the control and two experimental conditions. Significant ANOVAs were followed by a Tukey *post hoc* test and, when relevant, *P* values were given in the text

Source of variation	SS	Df	MS	F
GR_w	0.06	2	0.03	**16.03***
GR_l	0.02	2	0.01	2.56
SGR_w	0.60	2	0.29	**5.42****
SGR_l	0.03	2	0.83	0.43

Note: *$P < 0.0001$, **$P < 0.001$

A significant difference in SGRw was found between ΔpHΔT and ΔpHΔT + LAB tanks (Tukey HSD test, $P < 0.0001$). The GRw between ΔpHΔT and ΔpHΔT + LAB tanks exhibited a significant difference after 30 days (Tukey HSD test, $P < 0.05$). These results are in accordance with Robson et al. (2016) model showing that scallop growth increases with the increase in temperature. Seawater temperature is one of the most important factors affecting scallop growth in the wild (Kamermans and Saurel, 2022). In indoor aquaculture, growth rates increase with increasing temperature until a threshold is reached at which it is possible to observe a decline (Frouël et al., 2008). Moreover, increased growth, due to the LAB cultures, can mainly be attributed to exposure to temperatures at the optimum for the growth of scallops and bacteria culture in captivity (Čanak et al., 2023). Studies of the combined effect of temperature and pH on queen scallops are scarce. Zheng et al. (2022) found that ocean acidification reduced scallop growth. However, a possible explanation for the shift towards higher temperatures for growth at a lower pH may be that the bivalves cope better with higher temperatures than with a low pH. The long-term effects of lower pH on the growth of scallops are unclear. Since there is no previous research in this area, the results obtained are valuable data for further research in aquaculture in terms of climate change.

CONCLUSIONS

This is the first study to monitor the growth of queen scallop in terms of climate conditions as well as the first study to investigate the role of LAB in reducing the impact of climate change in aquaculture. The data obtained are a good basis for further testing and application of indigenous LAB on scallops and other commercial species under conditions of ecological valence. Bacteria *Lpb. plantarum* I adapted well and showed positive characteristics under stressful conditions. Thus, further research will be based on testing this strain and its role in the challenges related to shellfish mariculture under the conditions of climate change and in general. The results show that *A. opercularis* has satisfactory growth in captivity under climate change conditions, providing the possibility of aquaculture in the Adriatic Sea in the future.

The conditions of LAB ecological valence are challenging for shellfish farming due to the increase in temperature and decreased pH of seawater, and consequently higher pathogen growth. Existing methods of fish and shellfish protection that nowadays include the use of chemotherapeutics, antibiotics or vaccinations are neither fully effective nor environmentally friendly. Therefore, the protective use of LAB, especially the indigenous ones, can play an important role in controlling diseases and the growth of fish and shellfish in the future.

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UTJECAJ NADOPUNE PREHRANE S *Lactiplantibacillus plantarum* I NA ČEŠLJAČU *Aequipecten opercularis* POD SIMULIRANO PROMJENJENIM KLIMATKSIM UVJETIMA

SAŽETAK

U ovom radu ispitan je utjecaj dodatka *Lactiplantibacillus plantarum* I, autohtonog soja bakterija mliječne kiseline (BMK) prethodno izoliranog iz probavnog trakta kraljevske češljače *Aequipecten opercularis*, na mikrobnu populaciju i stope rasta navedenog školjkaša u uvjetima simuliranih klimatskih promjena (pH 7,8, T = 16 ± 2 °C). Nakon mjesec dana hranjenja, rezultati su pokazali zamjetno smanjenje patogenih mikroorganizama uz održavanje dovoljne količine živih stanica *Lpb. plantarum* I. Viši pH i temperatura rezultirali su većim stopama rasta, izmjerenom težinom i duljinom školjkaša, u usporedbi s kontrolnom skupinom. Dobiveni rezultati daju uvid na utjecaj dodatka BMK na rast školjkaša u normalnim i klimatskim promijenjenim uvjetima te omogućuju kontrolu patogenih mikroorganizama. U kontekstu klimatskih promjena, interakcije domaćin-patogen trebaju biti prepoznate i stavljen pod kontrolu primjenom prirodnih rješenja kako bi se smanjio utjecaj na okoliš.

Ključne riječi: bakterije mliječne kiseline, zakiseljavanje oceana, procjena rasta, akvakultura školjkaša, klimatske promjene.

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