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THE EFFECT OF FREEZE-DRIED *Lactiplantibacillus plantarum* I ON THE MICROBIOLOGICAL QUALITY OF QUEEN SCALLOP *Aequipecten opercularis*

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ABSTRACT

In this study, four selected strains of lactic acid bacteria of marine origin were freeze-dried using skim milk as a cryoprotectant. After freeze-drying, survival rates were determined under 24-hour exposure to seawater samples. Isolate *Lactiplantibacillus plantarum* I had the highest survival rate of 92.5% and was selected for further experiments. Freeze-dried *Lpb. plantarum* I strain was added to queen scallop *Aequipecten opercularis* (Linnaeus 1758) in circular basins under climate change conditions (temperature and pH modifications) for one month. After the feeding period, shellfish were collected and microbiological quality was determined for each scallop. The results indicate that the addition of *Lpb. plantarum* I significantly improved the microbiological quality of the cultivated scallops. The total number of bacteria together with *Staphylococcus* species was significantly reduced, and the added lactic acid bacteria strain was maintained at desired amounts during the entire feeding period. The results obtained indicate that the inclusion of *Lpb. plantarum* I as a dietary supplement could provide protection against pathogens and serve as a feasible approach to reduce infection levels when cultivating *A. opercularis* in captivity.

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INTRODUCTION

Marine aquaculture has emerged as a vital sector in addressing global seafood demand, prompting innovative strategies to enhance the health and quality of cultivated organisms. In this context, the utilization of lactic acid bacteria (LAB) has gained considerable attention for its potential to improve the microbiological quality and overall well-being of aquatic organisms (Chizhayeva et al., 2022). LAB are widely used in the fermentation of different foods (Wu et al., 2017; Zapaśnik et al., 2022), but their role in aquaculture has not yet reached its full potential. Some studies have monitored the impact of LAB from terrestrial sources (e.g. gastrointestinal tract of warm-blooded animals and humans or fermented food) on the prolongation of fish and shellfish shelf life as well as on its disease resistance (Cao et al., 2015; Iacumin et al., 2021). However, LAB of marine origin are still not fully investigated although they represent a better choice for marine environment animals since they are already adapted to this ecosystem and can better express their positive characteristics in comparison to terrestrial strains (Govindaraj et al, 2021; Zhang et al., 2022). As global climate patterns undergo shifts, the cultivation of marine organisms faces unprecedented challenges. Fluctuations in temperature and alterations in pH levels, attributed to climate change, necessitate innovative strategies to safeguard the health and sustainability of aquaculture practices (Maulu et al., 2021). LAB of marine origin can play a crucial role in supporting aquatic organisms to changes in temperature, pH and salinity by strengthening the immune system (Čanak et al., 2024). Expected mechanisms of action include creating a hostile environment for pathogens through the production of inhibitory compounds, competition for adhesion sites and essential nutrients, enhancement of host immune responses and modulation of interactions with the environment (Loh, 2017).

This study focuses on the freeze-drying of four strains of marine-origin LAB utilizing skim milk as a cryoprotectant and evaluates their survivability in the challenging marine environment. The subsequent phase of the study included the freeze-dried *Lpb. plantarum* I strain into the diet of queen scallop *Aequipecten opercularis* (Linnaeus 1758) under conditions simulating climate change-induced alterations in temperature and pH. As the shellfish were exposed to the probiotic supplement for one month, the impact on microbiological quality was examined.

MATERIALS AND METHODS

Shellfish

Samples of live *Aequipecten opercularis* (Linnaeus 1758), commonly known as queen scallops, were obtained 2 nautical miles southeast of the Albanež shoal in the E2 fishing zone (44°43'58.49" N, 13°56'48.94" E) at a depth of 49 m, using a fishing vessel and bottom-trawling net. The scallops (n = 160) were transferred to a tank with fresh seawater and transported to a flow-through tank (volume 190 L) at the experimental facility located at the Pula Aquarium for acclimatization. Water flow in the tank was maintained at 200 L/h. The scallops were fed daily with a live algae culture mix consisting of *Tetraselmis* sp. (Chlorophyta) (5×10^5 cells/mL), *Nannochloropsis* sp. (*Eustigmatophyceae*) (30×10^5 cells/mL), and *Phaeodactylum* sp. (*Bacillariophyta*) (12×10^5 cells/mL).

Microorganisms

Strains of lactic acid bacteria (LAB) used in this study (*Lactiplantibacillus plantarum* 1, *Lpb. plantarum* 2, *Levilactobacillus brevis* and *Lpb. plantarum* I) were isolated from the digestive system (intestine) of live queen scallops (*A. opercularis*), identified, and characterized in our previous research (Čanak et al., 2023). The strains are permanently deposited in the Collection of Microorganisms of the Laboratory for General Microbiology and Food Microbiology at the Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia.

Bacterial cultivation and freeze-drying

LAB strains were cultured in MRS broth (Biolife, Milan, Italy) at 37 °C overnight. Bacterial cells were harvested via centrifugation at 6440 rcf for 10 minutes (Z206A, Hermle Labortechnik GmbH, Wehingen, Germany), rinsed with sterile physiological saline and suspended in 10% skim milk. The initial number was modified for all strains and was 10^9 cells/mL. Prepared suspensions were frozen at -80 °C overnight. These frozen suspensions were subsequently lyophilized using a Christ Alpha 1-2 LDplus Laboratory freeze-dryer (Martin Christ, Osterode am Harz, Germany), and the number of viable cells was determined before and after lyophilization. The total viable count was determined using the spread plate method on MRS agar (Biolife), followed by an incubation period at 37 °C for 24–48 hours. By comparing the survival rates before and after lyophilization, the success of the process and the cells' ability to survive the lyophilization process were determined.

Survival of LAB strains in simulated seawater samples

After overnight growth in MRS broth (Biolife), investigated LAB strains were separated from the nutrient medium by centrifugation (6440 rcf), washed twice with sterile physiological saline and resuspended in simulated seawater (3.5% NaCl solution). Initial viable count and after 24-hour incubation at room temperature was determined using an indirect method of plating on MRS agar (Biolife), followed by incubation and expression of surviving bacteria as CFU/mL.

Experimental design

After one week of acclimatization, 60 scallops were divided equally between three separate tanks, each containing 190 L of seawater (30 individuals per basin). One basin served as a control and received only phytoplankton as food, while in the second experimental basin climate changes were simulated by increasing the temperature to 16 ± 2 °C and the pH to 7.8. In the third basin *Lpb. plantarum* I culture was added at a concentration of 5 × 10^2 cells/mL and climate changes were also simulated as previously described.

The cultures were maintained for 30 days under described conditions of temperature, pH and constant dissolved oxygen levels, which were monitored using a Hanna Instruments HI-98193 portable dissolved oxygen meter. During the six-hour morning feeding period, the water flow in the aquarium was intentionally restricted and regular siphoning was performed to remove excess food and waste. After one month, tissue samples were taken from ten scallops as composite samples and immediately stored at -20 °C for further analysis. Each sample was divided into two subsamples and all measurements were performed in triplicate.

Microbiological analysis of queen scallops

Conventional microbiological techniques were employed to assess the microbiological quality of the queen scallop samples obtained in the preceding experiment. Microorganisms were tested in accordance with ISO norms.

For each sample, 1 g was homogenized in 9 mL of sterile water and serially diluted before plating on selective media using the pour plate method for total bacteria count and the spread plate method for other bacteria. All analyses were conducted in triplicate.

Total aerobic mesophilic bacteria were enumerated after incubation on nutrient agar (Merck, Darmstadt, Germany) at 37 °C for 48 hours in accordance with HRN ISO 4833 method. *Enterobacteriaceae* were enumerated after incubation on Violet Red Bile Glucose (VRBG) agar (Biolife) at 37 °C for 48 hours (ISO 21528-1:2017). Sulfite-reducing anaerobes were cultured on iron sulfite agar (Biolife) at 37 °C for 48 hours (ISO/CD 15213-2), *Staphylococcus* spp. on Baird-Parker (BP) agar (Biolife) at 37°C for 24 hours (ISO 6888-1:2021), LAB on MRS agar (Biolife) as previously described, and *Vibrio* spp. on TCBS Kobayashi agar (Biolife) in accordance with ISO 21872-1:2017 method.

Microbial growth was quantified using traditional plate counting and the results were expressed as colony-forming units per gram of shellfish meat (CFU/g).

Statistical analysis

Statistica 9.0 (StatSoft Inc., Tulsa, OK, USA) was used for the statistical analysis. The data are shown as mean ± SD and the significant difference among the solutions used.

RESULTS AND DISCUSSION

In this study, the first step of the investigation was freeze-drying LAB strains of marine origin and checking their ability to survive in simulated seawater. The results are presented in Figure 1 and Figure 2.

The highest survival rate after lyophilization was observed for *Lpb. plantarum* I (93%), while other strains displayed a survival rate of less than 90% (Figure 1).

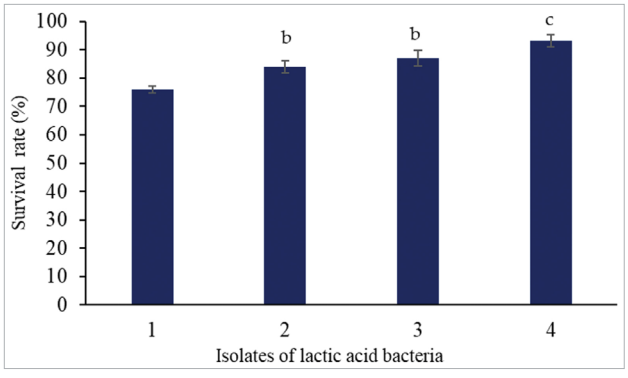

Fig 1. Survival rate of lactic acid bacteria strains during freeze-drying with skim milk as a cryoprotectant. LAB strains: 1: *Lactiplantibacillus plantarum* 1, 2: *Lactiplantibacillus plantarum* 2, 3: *Levilactobacillus brevis*, 4: *Lactiplantibacillus plantarum* I. [a,b,c] Different letters designate statistical difference at $P < 0.05$.

The results obtained are consistent with those of Reddy et al. (2009), where the survival rate of *Lactobacillus* strains was more than 90% in the lyophilization process in skim milk. The findings of G-Alegría et al. (2004) confirm that the survival rate depends on the strain and that the use of skim milk protects bacterial strains from lyophilization conditions. Variations in the surface properties of microorganisms as well as in the structure of the cell wall and membrane lead to different levels of strain resistance to lyophilization conditions (Capela et al., 2006).

To further assess the potential of selected strains for aquaculture application, survival in simulated seawater was determined. Cell survival was determined after 24-hour exposure to simulated seawater. Results are presented in Figure 2. The highest survival rate was determined for *Lpb. plantarum* I (1 log reduction), while other strains varied but exhibited more than 2 log reduction in number.

Three out of four strains in this study showed a significant reduction in number after exposure to experimental conditions. As the tested strains originated from the marine environment, they can tolerate simulated seawater conditions to some extent. Certain lactic acid bacteria demonstrate remarkable resilience by surviving harsh conditions, and previous research concluded that tolerance of LAB to higher salt concentrations is strain-dependant (Yalçınkaya, et al., 2019; Papadimitriou et al., 2023).

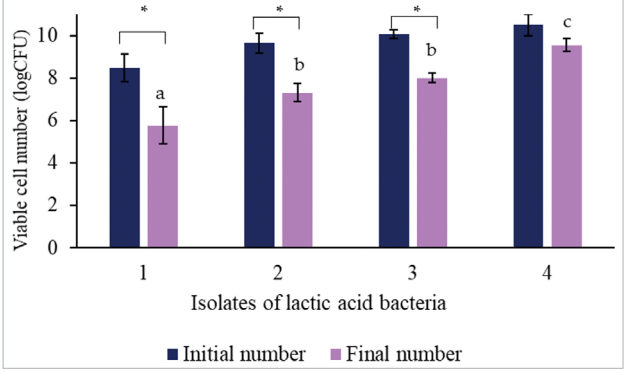

Fig 2. Effect of simulated seawater on lactic acid bacteria after 24-hour exposure. LAB strains: 1: *Lactiplantibacillus plantarum* 1, 2: *Lactiplantibacillus plantarum* 2, 3: *Levilactobacillus brevis* 4: *Lactiplantibacillus plantarum* I. [a,b,c] Different letters designate statistical difference at $P < 0.05$.
*Designates the difference between initial and final numbers for each strain.

Intracellular accumulation of organic osmolytes (glycine betaine, carnitine, choline) is considered the basis of the hyperosmotic stress response of LAB. These compounds can accumulate to molar levels without negative effects and are compatible with the macromolecular structure and function of the cell (Le Marrec, 2011). Also, with the aim of better determining the mechanism of LAB resistance to high osmolyte concentrations, the genomes of different LAB were sequenced and possible aquaporins, whose physiological role in osmoregulation has yet to be fully investigated, were identified (Derunets et al., 2024).
Since *Lpb. plantarum* I displayed the highest survival rate during lyophilization and the lowest reduction in numbers after exposure to simulated seawater, it was selected for further experiments.
The second stage of the experiments included estimating the impact of simulated climate change conditions in the absence and presence of *Lpb. plantarum* I on the cultivation of queen scallops. Results are presented in Table 1.

The highest number of bacteria was detected in the control sample, and simulating climate change conditions interestingly reduced the total bacterial count and *Staphylococcus* spp. This effect can be explained by the research of Cavicchioli et al. (2019) who concluded that species with limited ability to regulate their internal pH are likely to be more impacted by changes in environmental acidity. Factors such as organism size, aggregation state, metabolic activity, and growth rate will play an important role in determining this regulatory capacity. Lowering pH triggers shifts in gene expression in bacteria, prioritizing cell maintenance over growth. Ocean acidification is expected to reshape microbial food sources by affecting cellular growth efficiency, carbon cycling, and energy transfer (Bunse et al., 2016).
Although there are studies on the effects of climate change on marine animals (Sanderson and Alexander, 2020; Thorstad et al., 2021; Zgouridou et al., 2022), relatively few studies have examined the evolutionary adaptation of microorganisms to ocean acidification or other environmental variables relevant to climate change (Riebesell and Gattuso, 2015; Hutchins and Fu, 2017).
The addition of *Lpb. plantarum* I further reduced the number of mentioned groups of microorganisms, which is consistent with our previous research (Čanak et al., 2023) where the same LAB strain was added to the live bivalve in captivity in the form of wet biomass, while simulating conditions of ecological valence. Based on these results, it can be concluded that there is no difference in the state of LAB cells (lyophilized or wet biomass) as both inhibited pathogenic microorganisms. Their ability to produce antimicrobial compounds such as organic acids, hydrogen peroxide and bacteriocins lowers pathogen abundance by creating an environment unfavorable for growth (Ringø et al., 2020).
It is also important to note that the LAB number obtained at the end of the experiment (after 1 month) was relatively high at 10^4 CFU/g, indicating the possibility of adhesion and colonization of the intestinal system of shellfish, which is an important probiotic property. High concentration of

Table 1. Microbiological quality of scallops after one month of supplemented feeding with freeze-dried *Lpb. plantarum* I (CFU/g)

	Control	**Δtemperature ΔpH**	**Δtemperature ΔpH + *Lpb. plantarum* I**
Enterobacteriaceae	n.d.	n.d.	n.d.
Total bacterial count	2.6×10^8	5×10^5	1×10^4
Sulfite-reducing anaerobes	n.d.	n.d.	n.d.
Staphylococcus spp.	1.15×10^4	5×10^3	3×10^2
Lactic acid bacteria	n.d.	n.d.	1.14×10^4
Vibrio spp.	n.d.	n.d.	n.d.

n.d. – not detected

Lpb. plantarum I at the end of experiments also means that bacteria were present in sufficient amounts to express their positive effect throughout the experiment.

CONCLUSIONS

Introducing *Lpb. plantarum* I freeze-dried cells to the diet of queen scallops resulted in improved microbiological quality, indicated by a reduction in the total bacterial count and *Staphylococcus* spp., along with a relatively high LAB number after one month of cultivation under simulated climate change conditions. However, more studies are needed to fully understand the impact of climate change on microorganisms in marine environments and their adaptation mechanisms. The good survival rate of *Lpb. plantarum* I under these challenging conditions offers a basis for further research on LAB of marine origin as a potential solution for mitigating the consequences of climate change in fish and shellfish farming.

UTJECAJ *Lactiplantibacillus plantarum* I OSUŠENOG ZAMRZAVANJEM NA MIKROBIOLOŠKU KVALITETU ČEŠLJAČE *Aequipecten opercularis*

SAŽETAK

U ovom istraživanju četiri izabrana soja bakterija mliječne kiseline (BMK) morskog podrijetla osušena su smrzavanjem, koristeći obrano mlijeko kao krioprotektor. Nakon liofilizacije, ispitao se utjecaj morske vode na stopu preživljavanja tijekom 24 sata.

Izolat *Lactiplantibacillus plantarum* I imao je najveću stopu preživljavanja od 92,5% i odabran je za daljnje pokuse. Liofilizirani soj *Lpb. plantarum* I dodan je ishrani češljače (*Aequipecten opercularis* Linnaeus, 1758) tijekom mjesec dana, u recirkulacijskim bazenima pod uvjetima simuliranih klimatskih promjena (promjene temperature i pH).

Nakon završetka pokusa školjkaši su sakupljeni te je određena mikrobiološka slika. Rezultati pokazuju da je dodatak *Lpb. plantarum* I značajno poboljšao mikrobiološku kvalitetu kultiviranih češljača. Ukupan broj bakterija zajedno s vrstama *Staphylococcus* značajno je smanjen, a dodani soj BMK uspio se održati u zadovoljavajućim količinama tijekom cijelog perioda hranjenja. Dobiveni rezultati pokazuju da bi uključivanje *Lpb. plantarum* I kao dodatka prehrani moglo pružiti zaštitu od patogenih mikroorganizama i poslužiti kao moguć pristup za smanjenje infekcija tijekom uzgoja *A. opercularis* u zatočeništvu.

Ključne riječi: sušenje zamrzavanjem, uzročnici bolesti, češljača

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