J. Mar. Biotechnol. Immunol
Vol. 2 No. 1 (2024)
ISSN 3026-1457 (Online)
ISSN 3026-5274 (Print)

Journal of Marine Biotechnology and Immunology

Journal homepage : https://ejournal.immunolmarbiotech.com

Formulation of Ascorbic Acid and Skim Milk in Feed for Salinity Stress in *Artemia* sp.

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OPEN ACCESS

Article Info
Received: Desember 15, 2023
Accepted: January 19, 2024
Published: January 27, 2024
Available online: January 31, 2024

Keywords:
Artemia sp.
Ascorbic acid
Enrichment
Salinity Stress
Skim milk

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Abstract

Artemia sp. is recognized for its abundant and comprehensive nutritional profile, yet it lacks the inherent ability to produce vital elements such as vitamins, Eicosapentaenoic Acid (EPA), and Docosahexaenoic Acid (DHA), necessitating their acquisition from external sources. To augment the nutritional quality of *Artemia* sp., enrichment becomes imperative. This research aimed to evaluate the toxicity of ascorbic acid when used as a feed for *Artemia* sp. and investigate the influence of ascorbic acid enrichment and the addition of skim milk to the feed on *Artemia* sp.'s response to salinity stress. The study employed a Completely Randomized Design (CRD). The toxicity assessment of ascorbic acid utilized the Brine Shrimp Lethality Test (BSLT) method, yielding an LC50 value of 200.84 (> 1000 ppm), indicating toxicity to *Artemia* sp. Salinity stress resistance exhibited variability across treatments, with the longest survival times observed sequentially in *Artemia* sp. subjected to AS treatment 3 (20 hours), AS 2, and the control (16 hours), SM, AS 3, and C 1 (12 hours), and C 2 (8 hours). The enrichment of *Artemia* sp. feed with ascorbic acid and skim milk significantly influenced (sig. = 0.002) its resistance to salinity stress, with the optimal formulation identified at 200 ppm of ascorbic acid.

1. Introduction

Artemia sp. is utilized as a natural feed in aquaculture (Wibowo *et al*., 2013). It possesses growth factors, particularly proteins, which accelerate growth (Sorgeloos, 1985). The nutritional content of *Artemia* sp. includes 66% protein, 14% fat, essential amino acids, and fatty acids (Vajargah *et al*., 2021). The nutritional requirements of *Artemia* sp. for natural feed consist of 50% protein, 20% carbohydrates, 12-15% fat, 4% minerals, and 2-3% vitamins (Perdana *et al*., 2021).

Enriched *Artemia* sp. larvae can provide essential nutritional needs, enzymes, and other food elements (Gajardo and Breadmore, 2012). Ascorbic acid is effective in the formation of steroid hormones and enhancing the body's resilience to stress and infections. It plays a role in immune adaptation, biological activities, preventing body deformities, growth, and survival. Physiological factors such as stress resistance, toxicity, and immune activity in larvae of various species (Vajargah et al., 2021). Powdered milk is rich in carbohydrates, vitamins, fats, proteins, and essential unsaturated fatty acids, including EPA and DHA. *Artemia* sp. requires these fatty acids to meet the dietary needs of shrimp.

Toxicity testing is employed to understand the toxic effects of a compound. The toxicity test uses marine shrimp larvae or nauplii, and the Brine Shrimp Lethality Test (BSLT) method is cost-effective, simple, easy, and fast, providing reliable and representative results (Kurniawan and Ropiqa, 2021). *Artemia* can thrive in environments with seawater salinity, but juvenile *Artemia* may experience shock when transferred from higher salinity media to relatively lower salinity (Bwala et al., 2021).

The goal of enriching natural feed is to achieve nutrient composition close to the requirements for cultivation purposes. Enrichment of *Artemia* sp. can be done using ascorbic acid and skim milk. *Artemia* sp. can serve as a carrier for several nutrients. Slow growth and the immune resilience of an organism can be influenced by the lack of appropriate quality and quantity of feed. Enrichment with ascorbic acid and skim milk is a viable alternative to be tested for enhancing the resilience of *Artemia* sp. to salinity stress. The objectives of this study are to determine the toxicity level of ascorbic acid and assess the effects of ascorbic acid enrichment, skim milk, and the combination of ascorbic acid and skim milk in feed on the salinity stress of *Artemia* sp.

2. Material and methods

2.1 Materials

The materials used in this study involved the test organism *Artemia* sp. from the brand Supreme Plus, Great

Salt Lake. Ascorbic acid used in the research was sourced from the brand HL Vitamin, while skim milk used was from the brand NZMP MultiChem. The study was conducted at the Marine Biology Laboratory, Building H, Faculty of Fisheries and Marine Sciences, Universitas Diponegoro, Semarang, Indonesia.

2.2 Toxicity Test of *Artemia* sp. to Ascorbic Acid

The toxicity test on *Artemia* sp. was conducted using the Brine Shrimp Lethality Test (BSLT) method. Various concentrations of ascorbic acid, namely 100 ppm, 200 ppm, 400 ppm, 600 ppm, 1000 ppm, and a control, were administered to the test organisms. Observations were made at the 24-hour mark by counting the number of mortalities. Mortality data were processed to calculate the percentage of death, and the LC50 (Lethal Concentration 50%) value was determined using linear regression equations based on Mayer *et al*. (1982).

2.3 Research Methodology

This study employed an experimental laboratory method with a Completely Randomized Design (CRD). There were eight concentration treatments, each replicated three times. The enrichment of *Artemia* sp. was carried out through immersion using feed prepared with 25 ppt seawater. Treatments included ascorbic acid (AS) and skim milk (SM). Treatment doses included SM 600 ppm, AS 50 ppm, AS 100 ppm, AS 200 ppm, AS 25 ppm + SM 300 ppm, AS 50 ppm + SM 300 ppm, AS 100 ppm + SM 300 ppm, and a control.

2.4 Salinity Stress Test on *Artemia* sp.

After enrichment, *Artemia* sp. underwent a salinity stress test by transferring them from a 25 ppt salinity medium to a 0 ppt medium. Observations involved calculating the survival rate of the test organisms.

2.5 Data Analysis

Data analysis was performed using One Way Analysis of Variance (ANOVA) with SPSS 23 software and Microsoft Excel 2013. Normality and homogeneity tests were conducted as prerequisites for the ANOVA. If significant differences were found, further analysis was carried out using the Duncan test to detect significant differences among treatment groups.

3. Results

The toxicity test was conducted using ascorbic acid, and the results were utilized to obtain the LC50-24 hour value through linear regression analysis in Microsoft Excel. The test involved various concentrations of ascorbic acid, specifically 100 ppm, 200 ppm, 400 ppm, 600 ppm, and 1000 ppm. The toxicity results are presented in a regression analysis graph shown in (Figure 1). The toxicity test indicated an increase in the mortality rate of *Artemia* sp. with the escalating administration of ascorbic acid. The toxicity test results, calculated using Ms. Excel 2021 with probit analysis for LC50-24 hours, are presented in (Table 1). The composition of the skim milk used is suspected to influence the immune resilience of *Artemia* sp., and the skim milk has the composition presented in (Table 2).

The calculated LC50-24 hour value for ascorbic acid was 200.84 ppm. The probit analysis resulted in a coefficient of determination (R2) of R2=0.8364, meaning that 83.64% is controllable due to ascorbic acid being the variable under investigation.

The toxicity test using ascorbic acid was repeated three times for *Artemia* sp. at each concentration of 0 ppm, 100 ppm, 200 ppm, 400 ppm, 600 ppm, and 1000 ppm over 24 hours. The increase in concentration directly correlated with a rise in mortality, indicating a higher lethality. Figure 1 shows the probit analysis results of the toxicity test for ascorbic acid, yielding an LC50 value of 200.84 ppm.

Table 1. Analysis of Probit Data LC_{50}-24 Hours of Ascorbic Acid on Artemia sp.

Concentration (ppm)	Mortality (%)	Log Concentration (X)	Probit % Mortality (Y)	LC_{50} 24 hours (ppm)
100	3,33	2	3,34	
200	5	2,30	3,96	
400	15	2,60	8,12	200,84
600	100	2,78	8,12	
1000	100	3	8,12	

Table 2. Composition of Skim Milk (Brand: NZMP MultiChemp)

Type	Composition (%)
Lactose	54.5
Protein	32.9
Mineral	7.9
Moisture	3.8
Fat	0.9

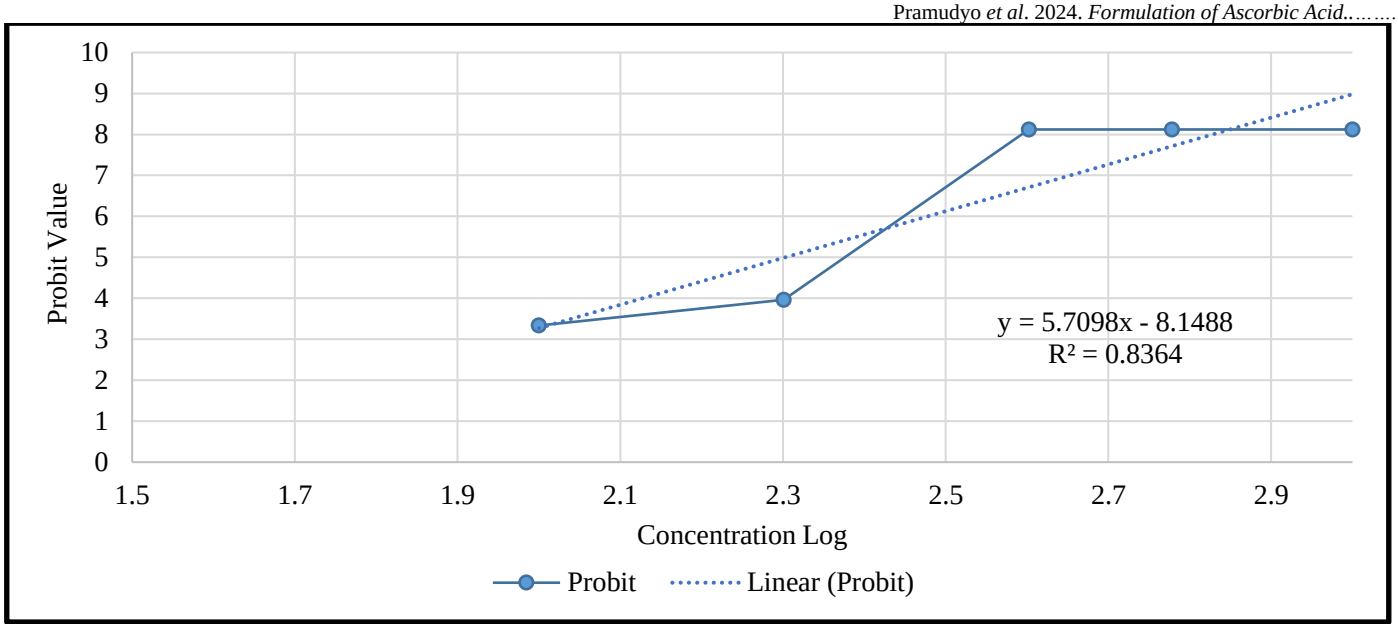
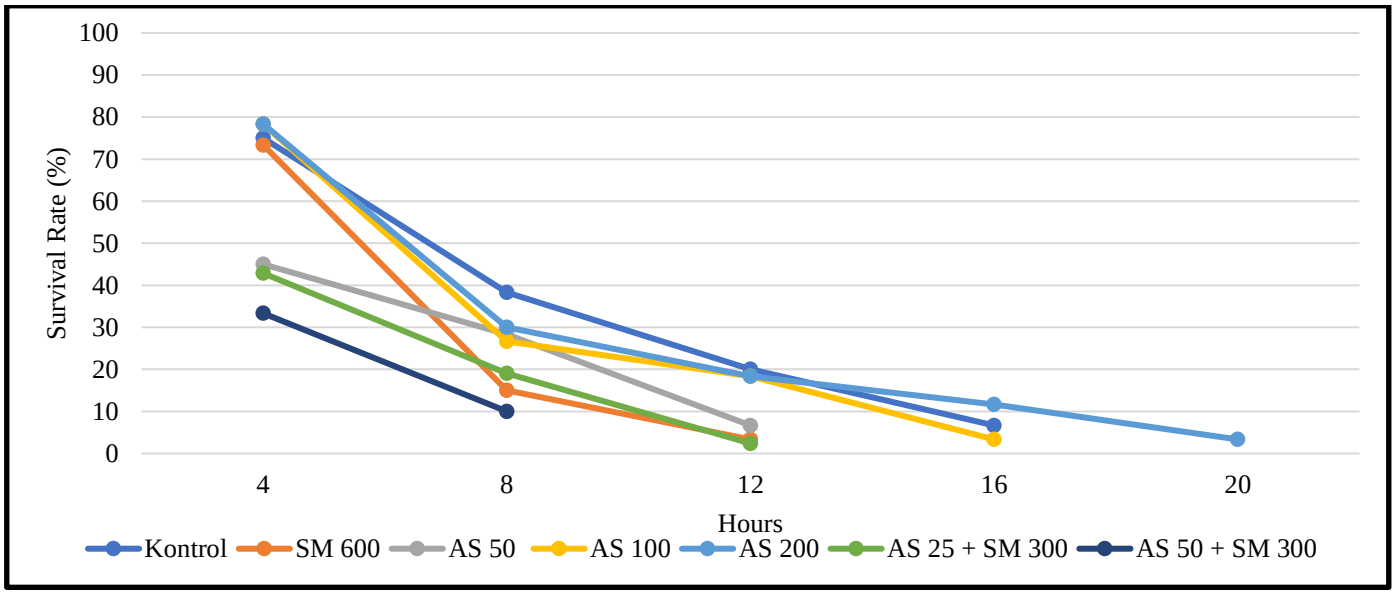
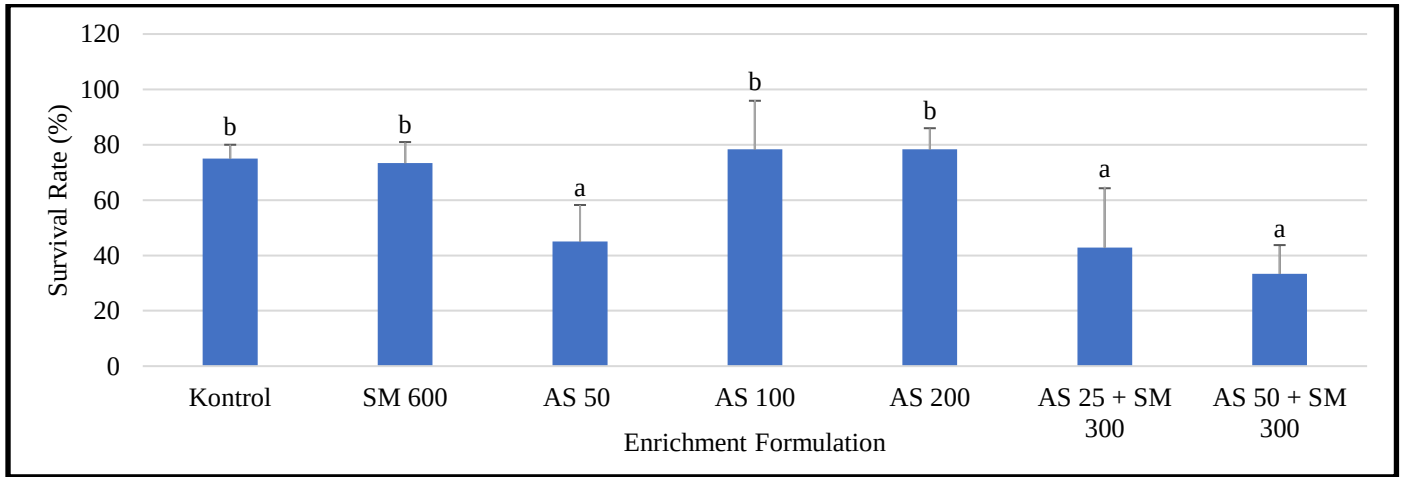


Figure 1. Regression Analysis of Log Concentration with Probit % Mortality of Ascorbic Acid.



Note : AS = Ascorbic Acid
SM = Skim Milk

Figure 2. Survival Rate of *Artemia* sp. under Salinity Stress Test at Different Time Intervals.



Note : AS = Ascorbic Acid
SM = Skim Milk

Figure 3. Salinity Stress Test with Feed Treatments on Survival Rate (Different notations indicate significant differences with a 95% confidence interval).

Observations of the survival rate of *Artemia* sp. during different times of the salinity stress test are presented in (Figure 2). The analysis of the salinity stress test on *Artemia* sp. after feeding with ascorbic acid and skim milk is shown in (Figure 3). *Artemia* sp.'s resilience to salinity stress at the 4th hour, in consecutive order, was AS 200 ppm, AS 100 ppm, control, SM 600 ppm, AS 50 ppm, AS 25 ppm + SM 600 ppm, and AS 50 + SM 600 ppm. The longest survival time of *Artemia* sp. was observed in the AS 200 ppm treatment for 20 hours, followed by AS 100 ppm and control for 16 hours, AS 50 ppm, SM 600 ppm, and AS 25 ppm + SM 600 ppm for 12 hours. In contrast, AS 50 ppm + SM 600 ppm exhibited the lowest resilience with a survival time of 8 hours. The one-way ANOVA analysis results indicated that the enrichment of ascorbic acid and skim milk in the feed significantly affected ($p<0.05$) the resilience to salinity stress in *Artemia* sp. Based on the observation data of *Artemia* sp.'s resilience to salinity stress, there were significant differences among treatments. AS 200 ppm was the treatment with the highest resilience and did not significantly differ from AS 100 ppm, control, and SM 600 ppm but significantly differed from AS 50 ppm, AS 25 ppm + SM 300 ppm, and AS 50 ppm + SM 300 ppm. The concentrations of ascorbic acid in consecutive order were AS 200 ppm, AS 100 ppm, and AS 50 ppm. The AS 200 ppm treatment emerged as the optimal concentration, as it exhibited the longest survival time under salinity 0 conditions compared to other treatments.

4. Discussion

The concentration with probit values indicates a linear correlation, with 16.36% of uncontrollable factors attributed to elements beyond ascorbic acid supplementation. Other factors, such as aeration, temperature, pH, salinity, and dissolved oxygen, are suspected to influence the life of *Artemia* sp. during the first three days (Bahari *et al*., 2014).

Hasan et al. (2021) stated that ascorbic acid possesses toxic properties. Meyer et al. (1982) classified ascorbic acid as having toxic potential with an LC50-24 hour value of 30 – 1000 ppm. Baiduri *et al*. (2018) highlighted the importance of ascorbic acid or vitamin C as a crucial nutrient. In crustaceans like *Artemia* sp., ascorbic acid is found only in the hepatopancreas and digestive tract.

The treatment with a high concentration of ascorbic acid enhances the resilience of *Artemia* sp. to salinity stress. Insufficient ascorbic acid may impair immune function, making larvae struggle to withstand stress. Ascorbic acid functions to normalize immune functions, reduce stress (Faidar *et al*., 2020), and prevent hormonal changes, maintaining the strength of the immune system as a potent biological antioxidant protecting cells from oxidative damage (Vajargah *et al*., 2021).

The composition of protein at 32.9% in skim milk is believed to contain amino acids. Protein molecules also contain phosphorus and sulfur. Phosphoprotein, found in milk casein, is utilized in *Artemia* sp. for emulsifying fats, serving as an energy source and essential fatty acid provider. Amino acids, including sulfur-containing ones like cysteine and methionine, play a crucial role in *Artemia* sp.'s growth by inducing protease secretion in the intestinal tract (Effendi *et al*., 2003).

The mineral composition at 7.9% in skim milk is suspected to contain micronutrients influencing *Artemia* sp.'s resilience to salinity stress. Potassium and calcium in skim milk significantly affect shrimp stress during salinity changes. The increased metabolic rate during stress reduces potassium levels in the body (Taqwa *et al*., 2016). The added mineral composition can replace reduced potassium and calcium levels, with iodine influencing *Artemia* sp.'s metabolism and overall health (Harefa *et al*., 2022).

The fat composition at 0.9% in skim milk is believed to contain n-3 HUFA, including EPA and DHA. DHA can alleviate various stress forms in fish larvae (Furuita *et al*., 1996). Higher concentrations of n-3 HUFA significantly improve larval development and provide better tolerance to salinity stress. n-3 HUFA, especially DHA, enhances shrimp resistance to osmotic shock, positively affecting their ability to combat stress conditions (Sui *et al*., 2007). The presence of DHA/EPA improves resistance to osmotic shock, with n-3 HUFA integrating into cell membranes and enhancing membrane permeability (Watanabe, 1993).

The enrichment dosage combining ascorbic acid and skim milk has a less favorable impact on the resilience of *Artemia* sp. at salinity 0. Treatments AS 25 ppm + SM 300 ppm and AS 50 ppm + SM 300 ppm are suspected to be toxic, indicating that ascorbic acid and skim milk do not synergize. Their mixture does not prolong *Artemia* sp.'s survival under salinity 0; instead, it may have adverse effects. This observation aligns with Supardan (2022), who emphasized that the metabolic process can either strengthen (synergize) or counteract (antagonize) the properties of a compound.

5. Conclusions

The toxicity test results obtained from the study reveal an LC50 value of 200.84 (> 1000 ppm), indicating that ascorbic acid exhibits toxicity towards *Artemia* sp. Enrichment with ascorbic acid and skim milk in *Artemia* sp.'s diet significantly influences (sig.=0.002) its resistance to salinity stress, with the treatment of 200 ppm ascorbic acid demonstrating the highest resilience.

Ethics approval

No need permit to *Artemia* sp.

Data availability statement

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

Credit authorship contribution statement

Virginia Hesa Febio Pramudyo: writing original draft preparation, visualization, project administration and data collecting. Sri Sedjati and Ervia Yudiati: Conceptualization, methodology, validation, investigation, resources, formal analysis, funding acquisition writing original draft preparation, writing review and editing, supervision, visualization. All authors have read and agreed to the published version of the manuscript.

Funding

No funding available.

Acknowledgments

I would like to state my special thanks of gratefulness to Diponegoro University.

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

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