

# Probiotic biosorption as a way to remove heavy metal in seawater

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## SUMMARY

**Industrial and feedlot water waste that contain large amounts of heavy metals can be harmful for Taiwan's aquaculture. This problem can be solved in an environmentally-friendly way by biosorption, which is using biological materials to absorb heavy metals. In this research, we aimed to reduce the food safety issues introduced by heavy metal contamination through the use of probiotics in food-producing aquafarms. *Lactobacillus rhamnosus* GG (LGG) and *Bifidobacterium longum* (BL) were added into seawater to determine if they have an ability to uptake heavy metal ions in a natural environment. Inductively coupled plasma mass spectrometry (ICP-MS) was used to detect the ion concentrations of arsenic (As), cadmium (Cd), and mercury (Hg) before and after biosorption. Both living and dead LGG showed a great biosorption capacity towards Hg and Cd, indicating that probiotics of LGG are a potential resource for efficient biosorption. Dead BL also showed a great biosorption capacity towards Cd and Hg, but much lower than living and dead LGG. Thus, the results of our experiment support the idea of using probiotics to solve heavy metal pollution issues in aquafarms in Taiwan.**

## INTRODUCTION

The aquaculture industry is very popular in Taiwan due to the country's island geography (1, 2), and this industry can greatly affect Taiwan's national economy and its citizens' health if the coastline is polluted. The amount of heavy metal in the ocean of the Taiwanese coasts is relatively high because of the industrial and feedlot water wastes (3), which results in damage to the ocean ecosystem and poisoning of aquatic organisms (4). Therefore, heavy metal pollution is a very serious issue that needs to be addressed.

Heavy metal ions comprise a large portion of industrial and feedlot water wastes (3). Releasing these heavy metal ions into rivers, estuaries, and the ocean severely damages the environment, eventually leading to the pollution of food sources. Consuming the polluted food can cause illness, even leading to death (5, 6). The most common heavy metal pollutants in the Taiwanese coastline are arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), nickel (Ni), lead (Pb) and zinc (Zn) (3, 7). Exceeding the legal standard of these heavy metal ions could lead to the abandonment of the aquaculture industry in several coasts of Taiwan. Among

these heavy metals, As, Cd, and Hg are easily taken up by marine organisms and very harmful to human beings (8).

There are several ways to clean up heavy metal pollution, including physical, chemical, and biological methods (9). Physical methods are more limited, and the solutions used to collect or dissolve the heavy metal could leave liquid or gases that are harmful to the environment. Chemical methods require a great amount of energy, and the chemicals used in the process can create waste products that will permanently damage the ocean environment and marine life. Therefore, using biological materials to absorb heavy metals is the preferred method due to its environmentally friendly nature and low cost (10).

The biological method of biosorption is ideal for solving issues within marine environments, since other methods' results might be disturbed by the high concentration of salt in sea water (11). Furthermore, biosorption can be performed using both living and dead cells (12), which greatly increases the capacity for heavy metal removal. The most common method of biosorption is bacteria-based biosorption, which offers several advantages (14). First of all, bacteria are able to uptake heavy metal in a few minutes as well as exhibit high binding capacities for metal ions. The bacteria can then be collected, effectively removing heavy metal ions from aquatic environments without byproducts. Secondly, bacteria grow at a very rapid rate; therefore, a great number of bacteria can be cultured in a short period of time, enhancing scalability. Lastly, if the recollected products leak out accidentally, the biosorption method will cause less burden to the environment compared to other methods. Considering these advantages, we think bacteria-based biosorption might be able to convert heavy metal polluted coastlines into healthy areas that are suitable for aquaculture in an environmentally friendly, time-efficient, and low-cost way.

Since we ultimately want to employ selected bacterial agents in industrial aquafarms, we chose organisms that have minimal impacts on human and environmental health. Probiotics, or bacteria and yeast that are considered beneficial for human health, are widely used in food products (14). Since probiotics are safely used in food production processes, they are suitable candidates for removing heavy metals from seafood within aquafarms. Probiotics have unique resistance mechanisms that allow them to bind and sequester heavy metals to their cell surfaces, removing heavy metals from the environment, without causing cellular damage (15). The most common probiotic organisms used are from the *Bifidobacterium* and *Lactobacillus* (16).

To quantify the concentration of ions in aqueous solution, several methods can be used. Two of the most common methods are atomic absorption (AA) spectroscopy and

**Table 1:** The removal efficiency of As, Cd and Hg for each bacteria group.

Group	As			Cd			Hg		
	BL (DA)	LGG	LGG (DA)	BL (DA)	LGG	LGG (DA)	BL (DA)	LGG	LGG (DA)
Removal Efficiency (%)	0.06	9.1	8.78	72.32	100	100	49.11	71.48	80.36

inductively coupled plasma mass spectrometry (ICP-MS). A common limitation for ion concentration detection instruments is that cannot be used on highly salinized samples. For example, with the AA spectrometer, salt can change the color of the flame, resulting in instability of the final detection. Also, the concentration of heavy metal in seawater is between 0.1 – 100 parts per billion (ppb), which is outside the detection limit of AA spectrometers. In contrast, ICP-MS is a highly sensitive instrument for detecting trace elements, and it is not as affected by high salinity (17). In addition, ICP-MS requires less volume of samples for detection. Therefore, we used ICP-MS which is often used to determine heavy metal concentrations.

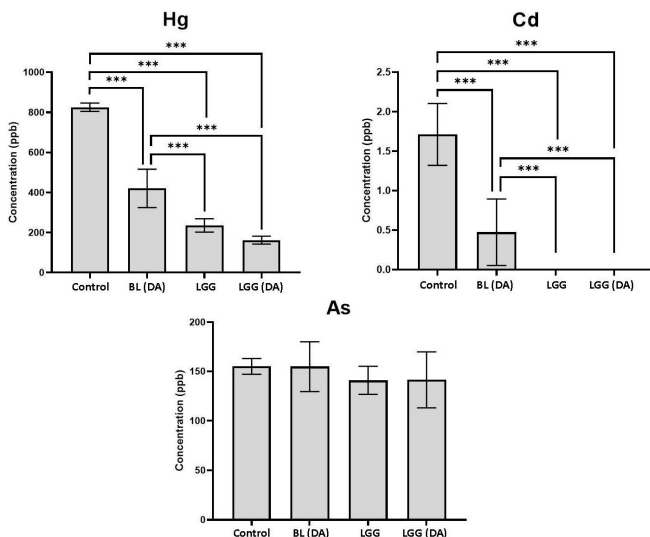
In this work, we tested the effectiveness of using probiotics, a biological material that is largely harmless to both humans and the natural environment, to reduce heavy metal concentrations in seawater. Our results supported our hypothesis that probiotics could clean up the heavy metal ions of seawater, which could have implications for providing an efficient way to maintain clean aquafarms that provide healthy and fresh food products to the public.

## RESULTS

For biosorption test, we incubated the probiotics into real sea water, and measured the concentration differential of metal ions between experimental group and the control. We purchased two types of probiotic supplements to obtain the

bacteria strains, which contained *Lactobacillus rhamnosus* GG LGG and *Bifidobacterium longum* BL bacteria powder, respectively, and tried to recover them in Lactobacilli MRS broth (MRS). The reason that we chose these two probiotics for this study is because they are the most commonly used probiotics in Taiwan. However, only LGG was successfully recovered, and BL did not expand in culture. The recovered LGG was separated into the LGG group and the LGG deactivated (DA) group, while BL only had the BL (DA) group. These deactivated groups were produced by heat-killing the cells. For this experiment, bacteria were used at a concentration of 1.25 g (7.6 x 10<sup>8</sup> colony forming units [CFU] for live LGG bacteria) per liter in each group in a total volume of 50 ml of seawater. The bacteria were left in the seawater for 2 hours prior to analysis. ICP-MS was used to detect the concentrations of remaining heavy metals, specifically of As, Cd, and Hg, in the seawater sample.

Following ICP-MS analysis, we calculated the removal efficiency of each group (Table 1) from the recovered ion concentrations (Figure 1). The concentrations of each ion are relatively different in the sea water of control group; there are 154.9, 1.7 and 825.7 ppb of As, Cd, Hg being detected. The best result for Cd biosorption was detected after 2 hours of incubation in the LGG and LGG (DA) groups, where both groups absorbed 100% of Cd ions (Table 1). For the BL (DA) group, only 72.32% of Cd ions were removed from solution (Table 1). The same resulting pattern was shown in Hg biosorption, where the LGG and LGG (DA) group had much better results than the BL (DA) group, with 71.48% and 80.36% removal efficiencies as compared to a 49.11% removal efficiency, respectively (Table 1). Across all groups, As biosorption was limited. Only the LGG and LGG (DA) groups absorbed a small amount of As ions after 2 hours of incubation, with 9.1% and 8.78% removal efficiencies, respectively. No substantial biosorption of As occurred in the BL (DA) group (Table 1).



**Figure 1:** The concentration of the As, Cd, and Hg in the sea water detected by ICP-MS. The control group is pure sea water without reaction. The BL (DA) group is the sea water treated with dead BL. The LGG group is the sea water treated with recovered LGG. The LGG (DA) group is the sea water treated with dead LGG. \*\*\**p* < 0.001 as measured by one factor ANOVA.

## DISCUSSION

In general, the results of our experiment support the hypothesis that probiotics can efficiently remove heavy metals from aquatic environments, and the result of our study supported this statement. Both of the probiotics have shown a good efficiency on cleaning the Cd and Hg ions in 2 hours of incubation.

In the experiment, while trying to recover both LGG and BL in MRS broth, BL recovery was not successful. One of the reasons for this occurrence might be because the organisms were deactivated during packaging by the manufacturer. Alternatively, BL may not be recoverable in a medium with oxygen, and a study by Dr. Shimamura in 1992 found a similar result (18), also MRS broth might not be the suitable medium for it. Our experiment could be extended by employing anaerobic equipment in the future.

Previous research suggested that dead probiotics showed better results in absorbing ions (19, 20). However, in this study, we found that the living probiotics also showed great results in absorbing ions (Table 1, Figure 1). Therefore, in addition to its known role as a probiotic, live LGG has great potential as a biosorption material.

Our experiment was completed successfully, and the results demonstrated proof of concept, but more work can be done. The method can be extrapolated to a larger scale situation more reminiscent of natural environments. For example, a logical next step is to greatly increase the amount of probiotics in the experiment, allowing the probiotics to have enough surface area to absorb the heavy metals of a whole aquafarm. Using microbial as soil amendment to improve the condition of arable land has been researched and commercialized for many years (21), however, there are less studies for marine aquafarms. Thus, we explored the possibility of aquafarm amendment by using the probiotics to improve the sea water condition, and we expect that this experiment could take place at one of Taiwan's many aquafarms for the future work.

## MATERIALS AND METHODS

### Bacteria

*Lactobacillus rhamnosus* GG and *Bifidobacterium longum* were cultured from LGG probiotic (Grape King Bio, Taiwan) and Suntory Lactobacillus Bifidus + Xylo-oligosaccharide (Suntory, Japan), respectively. They were seeded into Lactobacilli MRS Broth (NEOGEN, USA) for conducting biosorption in the seawater sample.

### Collecting seawater

A liter of seawater was collected from the southern coast of Shen'ao Fishing Port, Taiwan. The sample was collected by sinking a whole bottle vertically into the ocean. After collection of the seawater sample, it was filtered through 0.45 µm Millipore bacterial filters and then a 0.22 µm Millipore filter (Merck, Germany) into a new bottle in order to filter out insoluble matter and living creatures. The filtered sample was stored in the refrigerator at 4 °C.

### Culturing probiotics

All glass tools were wrapped in aluminum foil and, along with the MRS medium, placed into an EZ SS0-50E autoclave (EZ Medica, Taiwan) for sterilization at 121°C. Fifty milliliters of sterilized MRS medium with 3 g of probiotic powder were cultured at room temperature (RT) for 2 hours before subculture into new MRS medium overnight at RT. Bacteria were harvested by centrifugation at 4000 x g at 25 °C for 5 minutes (Centrifuge 5804 R with A-4-44 swing bucket rotor, Eppendorf, Germany), and the bacterial pellet was washed by Milli-Q water (MERCK, Germany) twice to remove the MRS medium. After cleaning, the bacteria were resuspended by Milli-Q water at a final concentration of 25 g per liter. The two deactivated (DA) experimental groups were incubated in a 100 °C water bath for 20 minutes. For calculating the number of bacteria, 100- and 10,000-fold diluted bacterial solutions were sprayed on the MRS agar plate and cultured at RT for overnight, then the colonies were counted to calculate the bacterial numbers. All the experiments described above were completed in a 1300 Series A2 biological safety cabinet (Thermal Scientific, USA) under aseptic conditions.

### Biosorption in seawater

Biosorption in seawater was processed by resuspending the bacterial pellet at a final concentration of 1.25 g per liter of bacteria in seawater and incubating at 25 °C for 2 hours. The reaction was stopped by filtering the solution through a 0.22 µm Millipore filter (Merck, Germany) after centrifugation at 4000 x g at 25 °C for 5 minutes. To stabilize the heavy metal, nitric acid (SHIMAKYU'S PURE CHEMICALS, Japan) was used with a final concentration of 1.7%.

### ICP-MS assay

An Xseries II ICP-MS instrument (Thermal Scientific, USA) was used in this experiment under the conditions described below. The forward power of the machine was set to 1250 W, and the gas flow rates were 14.5 L/min for cooling gas, 0.85 L/min for auxiliary gas, 0.89 L/min for nebuliser gas, and 4 L/min for CCT Gas. All the gases except CCT were helium instead of argon. Five concentrations of 3.125, 6.25, 12.5, 25, 50 ppb of standard ion were used to generate the standard curve. All the concentration results of detection have the detection limit of 0.01 ppb.

### Removal efficiency

Here we used a formula to calculate the removal efficiency, which is described as follows:

$$\text{Removal efficiency} = ((C_0 - C_t) / C_0) \times 100\%$$

$C_0$  was defined as the ion concentration before treatment, and  $C_t$  was defined as the ion concentration after treatment.

### Statistical analysis

All experiments were performed in triplicate. All statistical analyses were performed using Microsoft Excel and the Real Statistic add-in package. The difference in mean values were obtained using the Turkey HSD option in One-way ANOVA. All figures were generated by Sigma Plot.

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