The use of probiotic *Lactobacillus plantarum* and *Lactobacillus bifermentans* as antidote of mercury metal (Hg) to the mice *Rattus norvegicus*

Z Dwyana, Fahruddin, N Haedar, Ambeng, D Priosambodo and M R Alam

Department of Biology, Faculty of Mathematics and Natural Sciences, Hasanuddin University, Jl. Perintis Kemerdekaan Km. 10 Tamalanrea, Makassar 90245, South Sulawesi Indonesia.

Correspondence: zaraswatidwyana@gmail.com.

**Abstract.** Methyl mercury is a sort of most toxic mercury which was long since used by society in various fields. Methyl mercury is produced by a methylation process of anorganic mercury compound by metanogenic bacteria in aquatic sediment. The existence of excessive methyl mercury in the environment will be dangerous if entering the body of living beings. The aim of this research was to know the potential of probiotic bacteria spesies *Lactobacillus plantarum* and *Lactobacillus bifermentans* (*in vivo*) as antidote of mercury (Hg) poisoning to the experimental mice (*Rattus norvegicus* L.) after contacting methyl mercury acetate. This research used 5 various treatments where each of them conducted in 3 times repetition. The treatment which was used as follows; aquadest (positive control), mercury acetate 2 ppm (negative control), and probiotic bacteria *Lactobacillus bifermentans; Lactobacillus plantarum;* and also mixture culture of them. The observed parameter is the concentration of methyl mercury in blood and also through morphology observation qualitatively. The result of research shows that the giving of probiotic bacteria gives the real effect in decreasing the concentration of methyl mercury in blood. The best potential of probiotic bacteria as an antidote is *Lactobacillus bifermentans* because it can reduce the concentration of giving methyl mercury around 90.7 %.

1. **Introduction**

Human beings are threatened by dangerous chemical substances which are contaminated directly or indirectly every day. At least 50,000 pollutants in form of chemical substance contaminated the environment which cannot be avoided by human, then human have to realize the danger which was caused by them [1]. One of the contaminated materials in an environment which is quite dangerous is heavy metal. Heavy metal is difficult to be degraded, so the accumulation of metal ions can achieve the toxic concentration which can affect to the ecological damage. Heavy metal can also enter into the human body, like as; through respiration because inhale factory’s smoke, through foods which were contaminated by cooking tools, or enter through the cycle of the food chain in the environment. Mercury (Hg) or quicksilver is a heavy metal which is generally toxic and plays a part in pollution of
environment. Mercury toxicity depends on the form of chemical compound and the place of its contamination [2].

According to [3], anorganic mercury compound, particularly methyl mercury is the most excessive concentrated in the food chain. The fish consumed the plant which contaminated and made mercury to be accumulated in its body. The protein of fish bind strongly more than 90% methyl mercury, which was consumed, even though with cooking in a long time and strongly by frying, poaching or baking cannot detach it.

An organic mercury compound is more toxic rather than its anorganic compound because it is easy to penetrate the barrier of the brain’s blood and perfectly absorbed on the digestive duct. In accordance with [4], the beginning of toxic effect of methyl mercury occurred when its concentration on blood around 200-500 mg/ml. The minimum concentration of mercury in blood is around 30-50 mg/kg of body weight, this is equal with a daily need of 3-7 mg/kg. It needs to be noted that the emergence of mercury poisoning symptom can be delayed some weeks or months, depending on the accumulation of mercury compound in the body.

In pursuance of [5], the serious condition’s level of contamination will determine the trigger of sub-chronic toxicity effect and that toxicity occur if it is contacted in the lower concentration of chronic contamination. In this sub-chronic stage, the visible mark and symptom are the sense’s disturbance, constriction of visibility, suffering the deafness, and motoric disturbance.

The usual aid which is given to the victims of heavy metal toxicity is by giving an antidote. The antidote is a substance that can decrease or omit the compound of absorbed toxicity. Antidote can be produced from various chemical materials or biological microorganisms’ activities. The microbe, particularly bacteria can detoxificate mercury (Hg). Mechanism of heavy metal detoxification by the microbe goes on in a very complex way which includes precipitate and crystallization of heavy metal that occurs in the part of microbe’s extracellular and intracellular. Some of bacteria which have been known can resist toward mercury are facultativ and aerobic bacteria that conducted catalysis in the reduction process of Hg$^{2+}$ to Hg$^{0}$ like as bacteria Bacillus, Pseudomonas, Enterobacter, Corynebacterium, Micrococcus dan Vibrio [6].

The use of intestine’s micro flora in detoxification of heavy metal is excessive to be chosen by the researchers. This is because of its ability in binding metal at its cell wall, particularly, at gram positive bacteria, such as; Bacillus sp, Clostridium, Enterococcus and also Lactobacillus as the main group. These bacteria have the potential as probiotic and resist toward heavy metal. Probiotic is an intestinal microbe which can improve intestine’s micro ecology that gives a positive effect for the health of its host because its activity can restrain the pathogenic microbe of intestine and their carcinogenic activity [7, 8].

2. Material and Methods

2.1. Preparation of probiotic bacteria’s isolate

An isolate of probiotic bacteria included Lactobacillus plantarum and Lactobacillus bifermentans which were rejuvenated previously on the medium of MRSA around 1x24 hours. An isolate of bacteria were suspended by using physiological NaCl, then the density of their cell was counted until achieving 109 CFU/ml. An isolate of the probiotic Lactobacillus plantarum was encoded with probiotic LP and isolate of probiotic Lactobacillus bifermentans was encoded with probiotic LB. After that, they were ready to be given to the mice.

2.2. Making of mercury solution

Based on the value of mercury acetate LD$_{50}$ which was given to the mice per oral is around 41 mg/kg of body weight [9], that was equivalent with 4 ppm, then the dosage which was used is 2 ppm, that was obtained by dissolving around 0.002 g mercury acetate into 100 ml aquadest.
2.3. Preparation of experimental animal and the giving treatment
The mice were categorized into 5 groups of treatment, each of group consists of 5 mice, they are:
P1: variation which was given mercury solution with dosage 2 ppm;
P2: variation which was given mercury solution with dosage 2 ppm + suspension of probiotic LP;
P3: variation which was given mercury solution with dosage 2 ppm + suspension of probiotic LB;
P4: variation which was given mercury solution with dosage 2 ppm + suspension of probiotic LP and LB;
P5: variation which was not given any treatment.
Before giving the treatment, all mice were adapted with the condition of hutch and woof during 3 days. All groups of mice were given standard woof and drink. The volume of given probiotic solution and the mercury solution was based on the body weight of mice in each of the treatment’s group.
Probiotic solution for treatment’s group P2, P3, and P4 were given every day during 14 days orally. This is conducted in order probiotic bacteria can adapt in digestive-duct of mice. At 8th, 10th, and 12th days was given a mercury solution orally without stopping the giving of probiotic. Group P5 was not given any treatment. The clinical symptom observation about the physical condition of mice was conducted every day during 14 days.

2.4. Taking the blood samples
Taking the blood samples were conducted on the 15th days. The blood was taken through orbitalis vena at canthus medialis of the eye and was added into Eppendorf tube which has been contained with NaEDTA 1 %.

2.5. Calculation of mercury concentration in blood
2.5.1. Making prime solution of mercury. 1,3539 g HgCl₂ anhidrat was weighted, then dissolved into HCl 1 M and thinned until 1 litre.

2.5.2. Making standard solution. The prime solution of mercury was thinned by free mineral water to be standard solution 10 ppb, 25 ppb, 50 ppb, 100 ppb and 150 ppb.

2.5.3. Making reductor solution. Natrium borohidrid (NaBH₄) 0,75 g was dissolved into NaOH 1 % (b/v), then thinned until 100 ml with NaOH 1 % (b/v).

2.5.4. Destruction of sample. Blood sample NaEDTA was taken around 1 ml and added into a porcelain cup which has been weighted previously, then dried into oven until perfectly dry by using dry ice method. Dry sample which has formed powder was weighted around 5 g, and added into the three-neck flask. The sample was added with HNO₃ p.a. 65% and H₂SO₄ p.a. 96 % where each of them around 5 ml. Sample in the round bottom flask was connected with to the tool of modified Gorsuch. The sample was heated into temperature 60°C during 30 minutes. After that added HNO₃ 5 ml into the sample. The temperature was increased to 120°C, and then 150°C, heating process was conducted in heater mantel which was completed with thermometer. If sample’s color changes into black, then it was added H₂O₂ p.a. 30% drop by drop until the sample has clear color. After cold sample was filtered by filtration paper, then it was thinned until 50 ml by using free mineral water.

2.5.5. Analysis of mercury. At the beginning, Atomic Absorption Spectrophotometer (AAS) was turned on, after AAS ready to be used, the standard solutions which have been prepared with various concentrations, each of them was taken 5 ml and added 5 drops HNO₃ 1,5 % (v/v) and KMnO₄ 5 %, then added into the reaction bottle in MHS-10 tool which was connected on AAS tool Perkin Elmer 3110, then the absorbance was measured by 4 times repetition. For sample solution, it was also conducted by the same way in standard solution and also its absorbance was also measured.
3. Data Analysis
An analysis, which was used is limited quantitative analysis, then the body cannot do detoxification, then occur toxicity from the xenobiotic. It uses SPSS 20 program. Quantitative analysis was used for primary data observation, that is- mercury concentration in blood. The result of the experiment was analyzed with ANOVA (Analysis of Variance) and if there was any real difference between treatments, then will be continued with DMRT (Duncan Multiple Range Test) with significant standard 5%.

4. Result and Discussion
*Methyl Mercury concentration in blood after treatment*

The important factor which influences the potential of safety or not from a chemical substance is the relation between dosage of chemical substance with its appearing effect. Toxic property of chemical substance can be shown by the existence of functional, biochemical and structural change. Chemical substance can enter into the body through various ways. In this research, mercury acetate was given to experimental mice orally, thus, that substance will pass digestive duct, enter into circulation system, and then into the cell. In the normal condition, the body can eliminate xenobiotic through detoxification process, but if that xenobiotic dosage passes the limit, then the body cannot eliminate that xenobiotic.

The result of analysis of the average of mercury concentration in blood samples after giving probiotic can be seen in figure 1.

Based on Figure 1, the average mercury concentration in blood of white mice after 14 days treatment have the highest concentration rather than the positive control mice except the treatment group of *Lactobacillus bifermentans* probiotic.

The treatment group which was given *Lactobacillus bifermentans* probiotic has the average mercury concentration around 0.094 ppm. The treatment group which was given *Lactobacillus plantarum* probiotic has the mercury concentration around 0.321 ppm. The treatment group which was given *Lactobacillus bifermentans* and *Lactobacillus plantarum* probiotic has the mercury concentration around 0.429 ppm. While, the treatment group which was been as negative control and only given mercury without probiotic bacteria has the highest mercury concentration around 1.006 ppm. The group which was not given any treatment and was been positive control has mercury concentration around 0.203 ppm.

In 3 days, methyl mercury can be accumulated in the body because the existence of methyl mercury acetate’s binding with protein, polysaccharide, and amino acid. Besides that, methyl mercury acetate has a long half-time around 70 days and very few to be excreted [5].
The treatment group which was given probiotic bacteria that was compared with negative control group showed the decreasing of mercury concentration.

The negative control of treatment group which was compared with positive control group showed the increasing of mercury concentration around 0.803 ppm. The increasing of methyl mercury concentration in blood was caused by the absorption of methyl mercury acetate in digestive duct then transported into erythrocyte and plasma protein. Methyl mercury acetate is lipophil, can easily penetrate into cell membrane of the body and distributed into the body with the highest concentration in the central nerve system (more than 10% of total dosage), that is in organic form, but in the other tissue of the body, methyl mercury will be changed and saved in anorganic form of mercury with the highest concentration in liver and kidney [9]. With the reaction of oxidation-reduction, anorganic form of mercury will penetrate into erythrocyte, lungs, and liver in the form of divalent cation (Hg\(^{2+}\)). The giving of methyl mercury acetate showed the significant decreasing of mercury concentration. For the treatment group which was given probiotic bacteria species \textit{Lactobacillus bifermentans} decreased around 0.912 ppm. The treatment group which was given probiotic bacteria species \textit{Lactobacillus plantarum} decreased around 0.685 ppm. The treatment group which was given both probiotic bacteria species \textit{Lactobacillus bifermentans} and \textit{Lactobacillus plantarum} decreased around 0.577 ppm.

\begin{table}
\centering
\begin{tabular}{l l}
\hline
\textbf{Treatment Group} & \textbf{Methyl Mercury Concentration (ppm)} \\
\hline
Hg & 1.006100\textsuperscript{a} \\
(LB+LP) + Hg & 0.429667\textsuperscript{b} \\
LP + Hg & 0.320533\textsuperscript{b} \\
TP & 0.203300\textsuperscript{c} \\
LB + Hg & 0.094033\textsuperscript{c} \\
\hline
\end{tabular}
\caption{Methyl Mercury Concentration Based on The Result of DMRT Standard 5 \%}
\end{table}

Note: numeral which were followed by similar superscript alphabet in one column showed between quite similar treatment
Hg: The giving of methyl mercury acetate 2 ppm
TP: without any treatment
LB + Hg: The giving of probiotic \textit{Lactobacillus bifermentans} and methyl mercury 2 ppm
LP + Hg: The giving of probiotic \textit{Lactobacillus plantarum} and methyl mercury 2 ppm
(LB+LP) + Hg: The giving of both species probiotic \textit{Lactobacillus} and mercury 2 ppm

Based on the result of DMRT which was seen on Table 1 was known that all treatment groups were given different probiotic bacteria with treatment group which was only given mercury (negative control), indeed, methyl mercury concentration was higher because of there is no given microorganism which reduced that metal. The treatment group which was given probiotic bacteria species \textit{Lactobacillus plantarum} and the treatment group which was given culture combination of both probiotic bacteria (\textit{Lactobacillus bifermentans} and \textit{Lactobacillus plantarum}) were significantly different with the group without treatment (positive control). The treatment group which was given probiotic bacteria species \textit{Lactobacillus bifermentans} was not significantly different with the positive control group. Therefore, \textit{Lactobacillus bifermentans} has the best potential in detoxification heavy metal of mercury (Hg) in blood.

The group of lactate acid bacteria during this term includes in probiotic bacteria, for example at genus \textit{Lactobacillus} which has other function in the detoxification of heavy metal such as – mercury in an organism which has been contaminated by mercury [8]. This is related to biosorption mechanism. Gram positive bacteria have high absorbing capacity because of its contents – peptidoglican and teicoic acid, which were dominant at its cell wall, while gram negative bacteria has weak component and absorbents. In addition, the group of intestinal microbe has the role in conversing dimethylation of
mercury in the form of less toxic anorganic Hg^{2+} [8]. Therefore, it can be concluded that digestive duct has the potential intestinal micro flora to bind and penetrate heavy metal such as mercury.

One of the factors which can cause bacteria in having the resistance ability toward mercury is by existing of mercury’s resistance gene. The resistance bacteria toward mercury and has a mechanism to solve mercury’s spreading by the existence of resistance gene of mercury which was called gene mer-operon consists of metaregulator gene (merR), transported mercury gene (merT, merP, merC), reduction mercury gene (merA) and organomercuryliase gene (merB). merB gene encodes a catalyser which will catalyze dissolution of binding Me-Hg to be organic compound and ion Hg^{2+}, ion Hg^{2+} will bind to merC or merT. While Hg^{2+} at the outside of bacteria will enter to periplasma with pair sistein residue of merP. Then, merP transfers Hg^{2+} to sistein residue of merT or merC. Furthermore, ion Hg^{2+} penetrates to sitoplasma membrane through the reaction process of exchanging ligan to NADPH which depends on reduction mercury (merA gene). Reduction mercury gives 2 electrons to NADPH, therefore Hg^{2+} changes to Hg^0 which has volatile characteristic without producing energy for those bacteria. Furthermore, Hg^0 is excreted from cell.

According to [10], operon mer and operon ars are in bacteria genus Lactobacillus and species of other intestine normal flora which encodes eflux transporter. Bacteria which has the ability to export metal from cell can decrease damage of organism by reducing the cellular concentration. The ability of Lactobacillus to bind metal based on the resistance mechanism from its strain [8].

In fact, the result showed that the culture combination was not the best result because on this culture combination occurred competition between Lactobacillus bifermentans and Lactobacillus plantarum in consuming nutrition, therefore the function of Lactobacillus bifermentans was not too affect its ability to reduce heavy metal of methyl mercury (Hg).

5. Conclusion

Based on the result of research, it can be concluded that the giving of probiotic bacteria Lactobacillus bifermentans and Lactobacillus plantarum has the potential as antidote of mercury metal (Hg) with the best treatment is by giving probiotic bacteria species Lactobacillus bifermentans which can detoxicate heavy metal around 90,7%.

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