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RESEARCH ARTICLE OPEN ACCESS

Mercury Biodecontamination from Milk by using *L. acidophilus* ATCC 4356

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Abstract

Food and water contaminations with heavy metals have been increasing due to the environmental pollution. Decontamination of mercury as one of the most toxic heavy metals seems necessary. The aim of this study is to use *L. acidophilus* ATCC 4356 to reduce the mercury amount in milk. All possible process variables (including contact time, bacterial count, mercury concentration, temperature, contact time and shaking rate) were screening by Plackett Burman design for determination of main effects. Then main effects (contact time, as well as Hg and biomass concentration) were studied in 5 levels with response surface methodology to reach maximal bioremoval efficiency. The highest decontamination efficiency (72%) was achieved in the presence of 80 µg/L of initial Hg concentration, 1 × 10^{12} CFU of *L. acidophilus* ATCC 4356 in the 4^{th} day. Finally, the capacity of this bacterium for Mercury bioremoval was determined at different Hg initial concentrations by using the isotherm models of Langmuir and Freundlich. The results showed the higher correlation coefficient in Langmuir model so, Mercury absorptions obey Langmuir isotherm model. This study indicated that in the case of milk contamination to Hg, as reported in some countries, one of the solutions for metal decontamination could be the bioremoval by lactobacillus as natural valuable biosorbents as an environmental friendly technology.

Keywords: *Lactobacillus acidophilus*, Mercury, Biosorbent, Removal, Milk, Isotherm

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INTRODUCTION

Heavy metals with the density of more than 5 g/cm^3 are the essential elements for human body such as Zinc, Iron and Copper whereas some others are toxic even in very low amounts (in the range of µg/L) like Lead, Cadmium, Arsenic and Mercury[1]. There is an unwanted increasing in pollution of heavy metals into the air, water and soil and therefore food[2,3]. The sources of Mercury pollution are classified as natural resources (weathering of rocks, volcanic activities and biological processes), and the industrial activities (electricity power stations, mining, production of chemical, pesticides, cement, chlorine, mirrors, medical equipment and wastewater[4].

Milk is well-known all around the world for having vital effects on human health. The level of toxic metals is an important issue in quality and safety of milk[5]. Mercury is one of the toxic metals widely spread in the environment, water and food[6]. Mercury is recognized as human carcinogenic metal and produces gastrointestinal and immunological disorders[7]. The scientific food safety agencies are responsible for human health. Codex standard for contaminants and toxins in food has allowed the maximum permissible limits for mercury concentration in milk as less than 0.05 µg/L[8]. There are some reports of mercury contamination in milk in some countries like China 0.08 µg/L[9] and Iran, 0.07 µg/L[10].

Chemical and biological techniques have been used to eliminate heavy metals from polluted solutions. Chemical process like using rezin[11], ion exchange[12] and nanomaterials[13] but they are not efficient for low concentrations of the heavy metals and also are expensive and not environmentally safe.

Biological methods include using biosorbents like plants and microorganisms e.g. yeasts, bacteria, algae and fungi for bioremoval of heavy metal from food and water[14-16].

In the previous reports of this experimental team, we used *Saccharomyces cerevisiae* to remove heavy metals from water[17] and milk[18,19] but in this novel project the biosoption of mercury by *L. acidophilus* is evaluated as Lactic acid bacteria (LAB) are popular probiotics using all over the world.

The LAB have a desirable background of using in food processes in a safe manner as they are in the list of generally recognized as safe[15]. LABs have been reported to have the possibility in health applications and also bind the food contaminations like heavy metals and toxins even in low concentrations. Negative surface charge of LABs facilitates the binding to cations. So LAB would be a great microorganism for using in reduction heavy metals in water and foodstuffs[20,21].

LABs have been reported to remove the heavy metals bioadsorption of Cr by a novel Bacillus sp. CRB-B1[22], Hg bioremoval by *L. acidophilus*[6], biosorption of As by *Bacillus ferrooxidans*[23], Cd bioremoval by *Bacillus coagulans* and *L. plantarum*[24], Hg bioremoval by *B. cereus*[25], Se uptake by *L. acidophilus* [26] and As removal by *L. acidophilus*[27]. The gap of research in the previous reports, is the lack of an experimental design to evaluate all process variables for removal of heavy metal in the foodstuff and water (µg/L levels) instead of removal of heavy metal in wastewater (mg/L levels).

The aim of this study was to evaluate the capability of *L. acidophilus* ATCC 4356 for removing of mercury in milk in the range of µg/L. So, at first all possible process variables (including contact time, bacterial concentration, mercury concentration, temperature, contact time and shaking rate) were listed for a screening method of Plackett Burman design (PBD) and determination of main effects. Then, the main variables (contact time, as well as Hg and biomass concentration) have been studied in in 5 levels in response surface methodology (RSM) to reach the optimum condition for bioremoval efficiency. Finally, the capacity of *L. acidophilus* for Mercury bioremoval was determined at different Hg initial concentrations and also the biosorption isotherms were evaluate by using the two most famous isotherm models: Langmuir and Freundlich.

To our knowledge, there is no published study about the capability of *L. acidophilus* in biosorption of mercury in milk and this would be the first step of applying this valuable microorganism to remove the low levels (µg/L) of Hg concentration in milk successfully, therefore these results would open a new window in food decontamination by using this green technology for heavy metals removal in food industry.

MATERIALS AND METHODS

Reagents and chemicals

The standard solution of Hg $(NO_3)_2$ (1000 mg/L, Merck, Spain) and MRS agar, MRS broth and plate count agar were obtained from Liofilchem (Zona Industriale, Italy). The other chemicals were: nitric acid (Merck), phosphate-buffered saline (HyClone, Spain), H_2O_2 (Prolabo, Spain) and bovine serum albumin (Labclinic, Spain).

Bacterial strain and preparation

L. acidophilus ATCC 4356 as one of the most available and widely used probiotic was selected and prepared from Tak Gen Zist Company (Tehran, Iran). The bacteria were inoculated in MRS broth (10 ml), then incubated at 37°C for 48 h. The viability of *L. acidophilus* cells was evaluated by total plate counting and MRS agar and plate count agar used for *L. acidophilus* counting[28].

Sample preparation

The milk samples were designed according to the following schematic diagram (Fig. 1). Then the analysis was carried out through storage period.

Hg Analysis

The inductively coupled plasma mass spectrometer (ICP- MS) 4500a (England) was applied in this study, with a cross flow rate nebulizer and a Peltier-cooled quartz spray chamber. It was tuned up by an aqueous multi-element before each experiment. At first all the prepared samples were under digestion by using the microwave with segmented rotor MPR-600 (using pressure up to 35 bar; at 260°C)[29].

Plackett-Burman Design and selection of variables

The bacterial concentration, inoculation temperature, contact time, Mercury concentration and shaking rate are the effective independent variables on Mercury bioremoval by *L. acidophilus* as mentioned in previous studies[6, 30]. The variables were selected in this project by the help of literature reviews and pre-experience study (Table 1). Table 1 shows PBD for evaluation of 5 process variables in two levels.

The levels of Mercury concentration were selected by the aim of this project to study the potentiality of *L. acidophilus* to remove the low levels of Mercury (µg/L) in milk. Up to now there is no published information on this issue. For designed experiment of biosorption, the bacterial concentration (1×10^{11} and 1×10^{12} CFU) was added to sterile milk of 37°C in 250 mL Erlenmeyer flasks with the rest time of 20 minutes then Mercury (40 and 80 µg/L) added to the flasks. After that the flasks were put at on the shaker. At the contact time (1st or 4th day), bacteria cells were centrifuged at 8000 × g for 20 min. Then the supernatant was analyzed for residual Mercury concentration

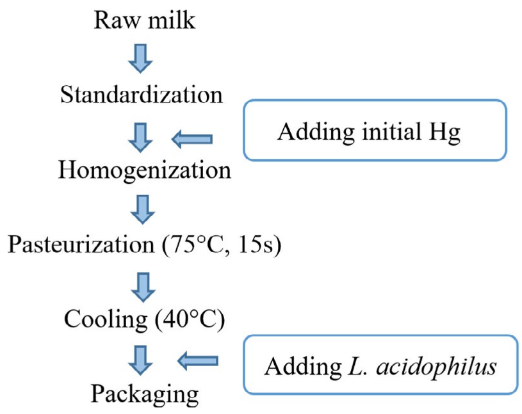

Fig. 1. Schematic diagram of milk samples production

Table 1. Plackett-Burman for evaluation of impact of the variable on Mercury biosorption by *L. acidophilus*

Run	Bacterial concen. (CFU)	Inoculation Temp. (°C)	Contact time (day)	Mercury concen. (µg/L)	Shaking rate (rpm)
1	1×10^{11}	40	4	100	0
2	1×10^{12}	4	1	100	50
3	1×10^{11}	4	4	100	50
4	1×10^{12}	40	1	100	0
5	1×10^{11}	4	1	40	0
6	1×10^{12}	4	4	40	0
7	1×10^{11}	40	1	40	50
8	1×10^{12}	40	4	40	50

by ICP- MS. All these experiments were carried out in triplicates. The ability of *L. acidophilus* to absorb Mercury was estimated by the following equation[31]:

$$\% \text{ Removal} = 100 \times [(C_0 - C1) / C_0]$$

Where C_0 is the initial and C_1 is residual Mercury concentration.

The data were analyzed using the Minitab (version 14) statistical software. According to the variance analysis, the 3 main variables were: Mercury concentration, *L. acidophilus* concentration and the contact time.

Response surface methodology (RSM)

RSM is the usage of statistical and mathematical techniques together for analyzing the independent variables on the responses. RSM is a helpful application in optimizing and improving the precess design. This method is more practical by applying interactive computer programs between the variables based on experimenter's prior knowledge. After all it represents the parameters effects on the process[32].

The Plackett-Burman results showed that 3 variables; *L. acidophilus* concentration, Mercury concentration and contact time, having significant effect on mercury bioremoval. RSM was designed for a completed determination of optimum variable levels for Mercury bioremoval and also elimination the tests number. In our project, CCD was used to find the optimal bioremoval conditions with the experimental factors levels displayed in the Table 2.

The other factors were kept constant as the following: the inoculation temperature at 25°C and the shaking rate at 50 rpm. For data analysis the Design- Expert 7.1.5 (Stat-Ease Inc., USA) software was used. The bioremoval runs were performed by the 5 levels of each variable (Table 2) and then the tests obtained by CCD.

Evaluation of the capacity of binding metal

The maximum capacity of binding Mercury would be predicted with the different isotherm models like Langmuir and Freundlich. The absorption models used for explaining absorption system of the bacterial cells in biosorption of metals at the contact time[33,34].

Statistical Analysis

The results of statistical analysis were done by MINITAB statistical software (version 14) and the response surface plots were prepared. The statistical data were provided by analysis of variance. All data are represented as the mean value ± standard deviation (M ± SD) of independent experiments in mentioned days. *P*-values below 0.05 were statistically significant.

RESULTS AND DISCUSSION

RSM for optimization of Mercury bioremoval

The analysis of variance showed the effect of the variables designed by Plackett-Burman Design. Using RSM after analysis of variance showed that the Mercury bioremoval level is the result of the 3 variables shown in Table 3. The P-values <0.05 showed that the model terms are significant. In this study Mercury concentration, contact time and biomass dosage are significant model terms.

Study the influencing factors on the effect of *L. acidophilus* on Mercury bioremoval

The factors influencing on biosorption of mercury by *L. acidophilus* in milk were analyzed and described below:

Effect of *L. acidophilus* concentration and contact time on removal efficiency

In this study, the experiments were done for evaluating the ability of *L. acidophilus* concentration in the range of 10^{10} to 10^{13} CFU on Mercury biosorption efficiency during the

Table 2. Main variables and levels for Mercury biosorption by *L. acidophilus* by central composite design

	Range and level				
Independent process variable	− α (−1.68)	-1	0	+1	+ α (+1.68)
L. acidophilus concentration (CFU)	1×10^{10}	10×10^{11}	1×10^{12}	10×10^{13}	10×10^{14}
Initial Hg concentration) µg/L)	40	50	70	90	100
Contact time (day)	0	1	2	3	4

contact times of 1 to 4 days. Fig. 1. shows the data collected from Mercury biosorption by *L. acidophilus* at different contact times. As it shows the maximum binding rate of Hg occurred in the 4th day. The removal efficiency of this heavy metal first enhanced with rising the bacterial concentration and contact time and reached to the maximum level and the further increase of bacterial concentration, caused a light decrease in removal level.

In general, heavy metals biosorption is a complicated mechanism. There are 3 theories in metal binding; the ion exchange with cell walls' teichoic acid and peptidoglycan, the precipitation and the ligands formation[20].

Lactic acid bacteria are gram positive and their cell walls contain a thick layer of teichoic acid, peptidoglycan and exopolysaccharides. The surface functional groups; carboxyl, hydroxyl, phosphate make the negative charges in *L. acidophilus*. So, the bacteria would be able to absorb the cationic ions of heavy metals[20,35].

The light decrease trend in removal process could be explain as a result of the bacteria partial aggregation at higher concentrations that causes the decrease in free sites in surface protein and exopolysaccharides and finally decreased biosorption[35,36]. In this study, the highest Mercury removal efficiency was 72% in biomass of 1×10^{12} CFU in the 3rd day. Similar studies reported the same results for increasing biosorption during exposure time by Halttunen[35] for Lactic acid bacteria, Rayes[37] for *L. rhamnosus* and *L. fermentum*. It has been reported that metal binding is a process carried out on the bacteria cell surface efficiently with no energy consumption[35].

As shown in Fig. 2. Mercury bioremoval enhanced by increasing the contact time from 1st to 4th day in addition to rising the bacteria concentration. The optimum level of *L. acidophilus* was 1×10^{12} CFU. It is sensible that by increasing the contact time, more Mercury ions would be connected to bacteria surface receptors and the

Table 3. Analysis of variance of parameters studied for Mercury biosorption by *L. acidophilus*

Source	Sum of Squares	df	Mean Squares	F-value	p-value
Shaking rate	0.211	1	0.211	2.88	0.4125
Mercury concentration	9.87	1	9.87	94.02	0.0542
L. acidophilus concentration	9.38	1	9.38	78.35	0.0712
Contact time	9.80	1	9.80	82.99	0.0688
Inoculation temperature	0.382	1	0.382	3.42	0.3202

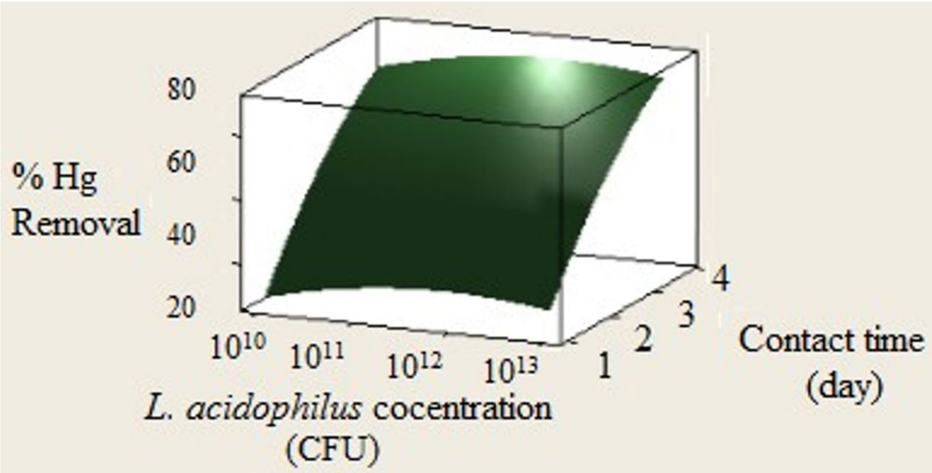

Fig. 2. Contour plot showing interactive effect of *L. acidophilus* concentration dosage and contact time on the Mercury removal

Table 4. Langmuir and Freundlich isotherm parameters of Hg in various initial concentrations

Hg initial (µg/L)	Langmuir model			Freundlich model	
	Ce	Qe	Ce/Qe	Ln Qe	Ln Ce
20	10.5	9.4	1.128	2.241	2.361
40	18	22	0.818	3.091	2.890
60	22	38	0.563	3.648	3.073
80	24	56	0.429	4.025	3.178
100	25	75	0.333	4.317	3.219

bioremoval process would be more efficient by time.

Effect of *L. acidophilus* concentration and Mercury concentration on removal efficiency

The effect of *L. acidophilus* concentration and initial Mercury concentration on the biosorption was investigated in the range of 10^{10} to 10^{13} CFU and 40 - 100 µg/L, respectively (Fig. 3.). The results revealed that by increasing the Mercury concentration, the adsorption increased. As shown in Fig. 3. Increasing *L. acidophilus* concentration up to 1×10^{12} CFU, make the removal efficiency enhancing. The maximum Mercury removal efficiency (72%) was observed at the initial Mercury concentration of 80 µg/L and the biomass concentration of 1×10^{12} CFU. *L. acidophilus* shows a high affiliation for biosorption of heavy metals[35,38]. Heavy metals binding is a surface process as the presence of anionic functional groups and it also depends on the capacity of the bacteria strains and the metal electronegativity[20]. It is reported that metal absorption in bacteria cells could be explained by the interactions between the heavy metals and the negative charge of bacteria surface. Gram positive bacteria like *L. acidophilus* have some polymers like lipoteichoic acid in their cell wall that can be responsible for such interactions[20,39].

It is observed that by rising the metal concentration, the absorption to the bacteria receptors would also increase, which results in the higher bioremoval level[38,40].

According to our findings Mercury biosorption efficiency increased by increasing the Mercury concentration in the range of 40 to 100 µg/L. The important factors as shown in Fig. 3, are Mercury concentration and the *L. acidophilus* concentration for Mercury bioremoval and their optimum levels are 80 µg/L and 1×10^{12} CFU for the maximum level (72%) of the biosorption. The same results were reported by Dobrowolski[40], Allam[41], Akhmetsadykova[42] and Halttunen[35] as the absortion would improve by increasing the bacterial concentration. Also by increasing the metal concentration, the biosorption would enhance as mentioned in some studies by Massoud[18] Halttunen[35], Shameer[36], Kinosita[38].

Isotherm model studies

The capacity of *L. acidophilus* concentration (10^{12} CFU/mL) for Mercury bioremoval was determined at different mercury initial concentrations (20, 40, 60, 80 and 100 µg/L). The biosorption isotherms are detemined

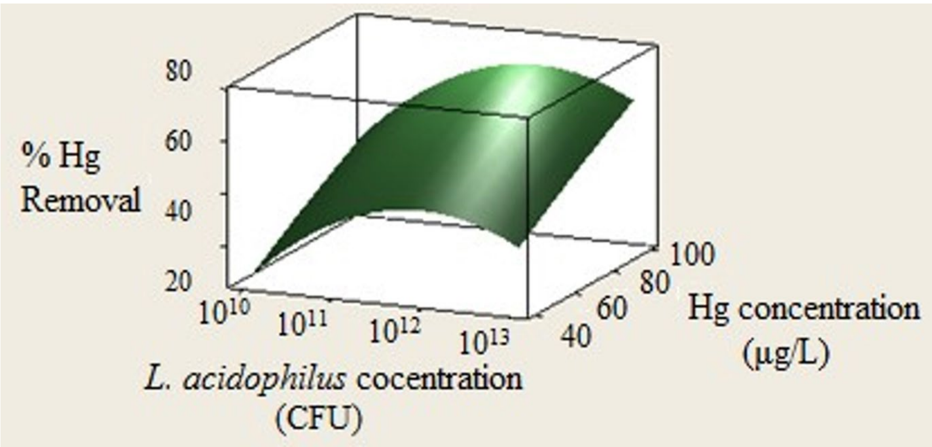

Fig. 3. Contour plot showing interactive effect of *L. acidophilus* concentration and Hg concentration on the Mercury removal

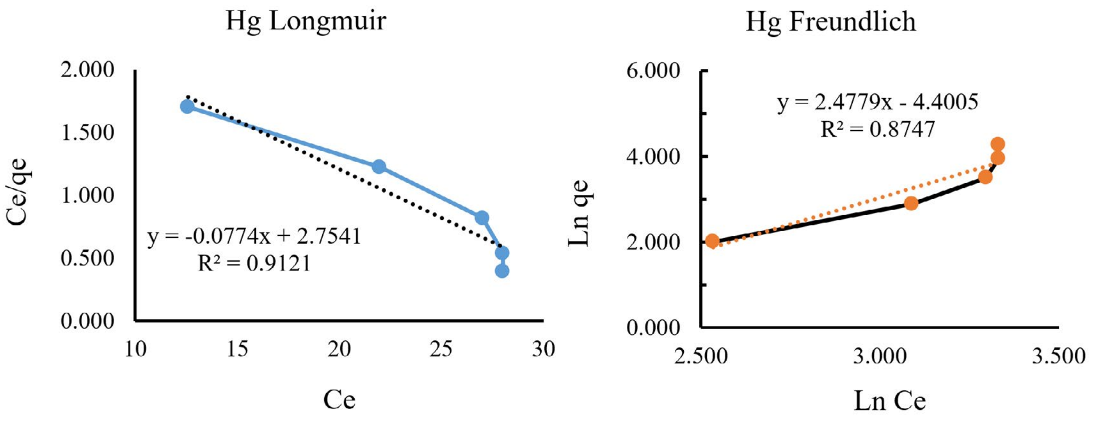

Fig. 4. Langmuir absorption isotherm curve (A), Freundlich absorption isotherm curve (B)

by using the isotherm models such as Langmuir and Freundlich. The regression coefficient (R^2) show the best isotherm describing the Mercury biosorption by *L. acidophilus.* All the experiments were performed in three replications.

The Langmuir model is the most common model used in scientific studies. The Langmuir equation is correct for monolayer absorption using the following equation[33]:

$$C_e / Q_e = 1/ (K_* Q_{max}) + C_e / Q_{max}$$

Where Q_e (µg/L) is the amount of Hg in absorbing equilibrium, C_e (µg/L) is the equilibrium concentration of Hg in milk, Q_{max} (µg/L) is the maximum Hg absorption in high C_e level; and K_L (L/µg) is the Langmuir constant. The C_e/Q_e versus C_e indicate a straight line of slope $1/Q_{max}$ and also intercept of $1/K_LQ_{max}$.

The Freundlich equation is as the following equation[34]:

$$\text{Ln } Q_e = \text{Ln } K_f + 1/n \text{ Ln } C_e$$

Where K_f and n is the Freundlich constants. The parameters K_F and n is defined from the linear plot of ln Q_e versus ln C_e. Freundlich equation varies with the materials heterogeneity. The Langmuir and Freundlich models' parameters are given in Table 4.

As shown in Fig. 4. A and B, the biosorption enhanced by increasing the initial Mercury concentration, because more metal concentration supplied more possible ions of Mercury ions to bind with absorbents' functional groups[6,43]. By comparing the both R^2 values in Langmuir and Freundlich models, it was inferred that Langmuir isotherm model showed better fit than Freundlich model, which also confirm that Freundlich equation is correct for monolayer absorption on surface binding. The higher correlation coefficient in Langmuir model indicates the Mercury absorptions obey Langmuir isotherm model.

CONCLUSION

The mercury presence in water and food is a public health problem. The European Rapid Alert System for Food and Feed (2018)[44] have reported that heavy metals are the contaminants that attracts the high notifications in water and food and Lead, Cadmium and Mercury are the ones that make the most problems for people. Among all the food and drinks, milk is the most sensible one that should be safe enough to be consumed.

In this project, RSM was used to evaluate the optimal condition for Mercury bioremoval by of *L. acidophilus*. Our findings showed the highest level of Mercury bioremoval of 72% in the concentration of 1×10^{12} CFU, the Mercury concentration of 80 µg/L and in the 4th day. The biosorption increased by increasing the metal and bacteria concentration as well as the contact time. This study represented the ability of *L. acidophilus* for Mercury removal in very low concentration levels (µg/L) from milk. Also, these findings open the doors of investigating the capacity of Mercury binding by LABs in milk. Further studies are suggested for other LAB strains in milk and foodstuffs to reduce the toxic effects of the heavy metals.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors have made substantial contribution to the work and approved it for publication.

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ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

DATA AVAILABILITY

Not applicable.

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