Short Communication

Probiotic *Lactobacillus* sp. with bioremediation potential of toxic heavy metals

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Pollution of the environment and food with toxic heavy metals is being intensified in Bangladesh with industrial development. Consumption of foods and water contaminated with heavy metals imposes threat to human health. Aiming to find a solution to this problem, the present study focuses on probiotic *Lactobacillus spp.* with potential to remove heavy metals from environment as well as human body. A total of three *Lactobacillus* spp were isolated from curd samples and were identified based on their morphological and biochemical properties. These isolates were tolerant to low pH and bile salt which aids in their application in human gut. All isolates could tolerate 600 ppm chromium, 400 ppm lead, 400 ppm copper and 400 ppm zinc. The heavy metal tolerant *Lactobacillus* spp were also multi drug resistant and showed 100% resistance to Azithromycin, Cloxacillin, Gentamicin, Vancomycin, Streptomycin, Nalidixic acid, Trimethoprim-Sulfamethoxazole and Penicillin, while 100% sensitivity was observed to Imipenem.

Key words: *Lactobacillus*, heavy metal, antibiotic

Heavy metal contamination is a serious problem from both human health and environmental perspective. The increasing contamination of food and water with a range of heavy metals, such as lead, cadmium, arsenic, chromium, and mercury and their exposure to populations remains a major concern in Bangladesh in recent years[1]. These hazardous, toxic heavy metals adversely affect human health by bioconcentration, bioaccumulation and biomagnification phenomenon. In children, exposure to high concentration of heavy metals like cadmium, lead, chromium, mercury can cause potentially permanent learning and behavior disorders[2]. In adults as well as in children heavy metals have deleterious effects on heart, bones, intestines, kidneys, reproductive and nervous systems and leads to death in extreme cases[2]. While many developed countries have taken some actions to monitor and reduce the problem of heavy metal contamination, it remains neglected in Bangladesh. It is worsening with increasing urbanization and industrial development. Additionally, available conventional methods (precipitation, flocculation, ion exchange, and membrane filtration) for removal of heavy metals from the environment are sometimes inefficient and expensive for practical large-scale use, especially for developing countries like Bangladesh[3]. Therefore it is necessary to seek alternative solutions for reducing the load of toxic heavy metals from the environment and thereby protecting human.

Recently, ability of probiotic bacteria, such as *Lactobacillus* sp., to bind and detoxify toxic heavy metals has generated much attention globally[4-9]. Moreover, with evidences of safe application in human, *Lactobacillus* becomes an attractive candidate for preventing absorption of metals into the human body[6]. Use of Lactobacilli also offers some advantages, such as, immediate application, no requirement for expensive technology or infrastructure setup etc[7, 9]. Therefore, this bacterium can also be used as a tool to reduce heavy-metal toxicity from environment[7, 9]. This study aims to isolate heavy metal-resistant *Lactobacillus* sp. from yogurt samples, which could potentially be used for removing heavy metals from environment as well as human health.

To isolate *Lactobacillus* sp. five different curd samples were collected from shop and 0.1 ml from each curd sample was spread on to MRS (De Man, Rogosa and Sharpe, Difco Laboratories, Detroit, MI, USA) agar medium, a selective medium for *Lactobacillus*. Following incubation at 37°C for 24 hours isolated colonies were selected for further characterization. Presumptive *Lactobacillus* spp, were identified using microscopic analysis and biochemical tests as suggested by the Bergey's Manual of Bacteriology[10].

To determine the maximum tolerance of the *Lactobacillus* isolates to heavy metals, each isolate was grown at 37°C for 48 hours in 5 ml MRS broth medium in screw capped tubes supplemented with different concentrations of each metal (lead, chromium, copper or zinc). Stock solutions of lead as 0.5M $Pb(NO_3)_2$ and chromium as 4.0M $K_2Cr_2O_7$ were prepared and filter sterilized. From each stock solution, appropriate amount was added in MRS broth to dissolve lead in the concentration ranging from 100 to 500 ppm and chromium ranging from 100 to 600 ppm. Analysis of resistance to copper and zinc was also determined in a similar way. Copper was added to MRS broth as $CuSO_4.5H_2O$ (100-500 ppm) and zinc was added as $ZnSO_4.7H_2O$ (100-500 ppm). Following incubation at 37°C for 48 hours,

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growth of each bacterial culture was measured by measuring absorbance at 600 nm.

The acid-tolerance of the *Lactobacillus* isolates was investigated following the method described by Bhakta *et al.,* (2012)[9]. Briefly, fresh culture of *Lactobacillus* sp. grown in MRS broth was centrifuged at 13000 g for 5 min and the cell pellet was washed twice with normal saline. The cell pellet was then suspended in sterile phosphate-buffered saline (PBS) adjusted to pH 1.0, 1.5, 2.0, 2.5 or 3.0 (using 10M HCl) and incubated at 37°C for 2 h. Following incubation, 0.1 ml bacterial culture in PBS was inoculated on MRS agar medium and tolerance of the isolates was assessed in terms of their growth after 48h incubation at 37°C.

Bile-tolerance of the *Lactobacillus* isolates was studied using the method described by Bhakta et al., (2012)[9]. Each isolate was grown at 37°C for 24 h in MRS broth supplemented with bile salt (Sigma-Aldrich, Germany) at concentrations of 1000, 2000, 3000, 4000, 5000, 6000, 7000 and 8000 mg/l). After 48h incubation at 37°C, the growth of isolates was recorded by measuring absorbance at 600 nm.

Bacterial susceptibility to anti-microbial agent was determined *in vitro* by standardized agar-disc diffusion method known as the Kirby Bauer method using commercial antibiotic discs (Oxoid, UK)[11]. Antibiotics and disc potencies used were Penicillin G (10 µg), Chloramphenicol (30 µg), Azithromycin (15 µg), Cloxacillin (5 µg), Gentamicin (10 µg), Vancomycin (30 µg), Streptomycin (15 µg), Nalidixic acid (30 µg), Trimethoprim-Sulfamethoxazole (25 µg) and Imipenem (10 µg).

Based on colony morphology on MRS agar, Gram staining and catalase test three presumptive *Lactobacillus* spp. were isolated from five different curd samples (Figure 1). Further identification at species level needs to be carried out by 16s rRNA sequencing.

The *Lactobacillus* isolates were investigated for their tolerance to different concentrations of heavy metals chromium, lead, copper and zinc. All isolates were able to grow at 600 ppm concentration of chromium and could tolerate 500 ppm concentration of lead, copper, and zinc (Figure 2). This data indicate that these isolates can easily survive in high heavy metal contaminated environment and they might be useful as potential agent for heavy metal bioremediation purpose. However, with increasing concentration of each heavy metal, growth of each isolate decreased. The slower growth rate might be because of the fact that the *Lactobacillus* spp. need to spend more energy on activation of mechanisms and pathways that allow tolerance of the metals.

Human intestine and stomach is characterized by high acidity and high concentration of bile salts. Therefore acid- and bile-tolerant abilities are the two primary important criteria for the establishment of any bacterium into the human intestine and stomach (Hyronimus *et al*. 2000). All three *Lactobacillus* isolates sp. obtained in this study were found to tolerate up to 8000 mg/L bile salt (Figure 3). None of them grew at pH 1.0, while only isolate 1 was able to grow at pH 2.0 and pH 3.0. All isolates showed best growth at pH 2.5 (Table 1). These observations suggest that these isolates are perfectly suited for administration in human gut for detoxification of heavy metals, of which isolate 1 possesses a wider pH range for growth compared to the other two.

Several studies suggest that antibiotic and heavy metal resistance genes are often encoded together on the same plasmid[13, 14]. Therefore, the heavy metal tolerant *Lactobacillus* isolates were tested for their antibiotic resistance profile. Investigations revealed that all three isolates were highly resistant to commonly used antibiotics with some exception. All isolates were sensitive to Imipenem only and were resistant to the other antibiotics tested (Azithromycin, Cloxacillin, Gentamicin, Vancomycin, Streptomycin, Nalidixic acid, Trimethoprim-Sulfamethoxazole and Penicillin) in varying degrees (Table 2). Isolate 2 showed more resistance to the antibiotics than the other two isolates. Similar
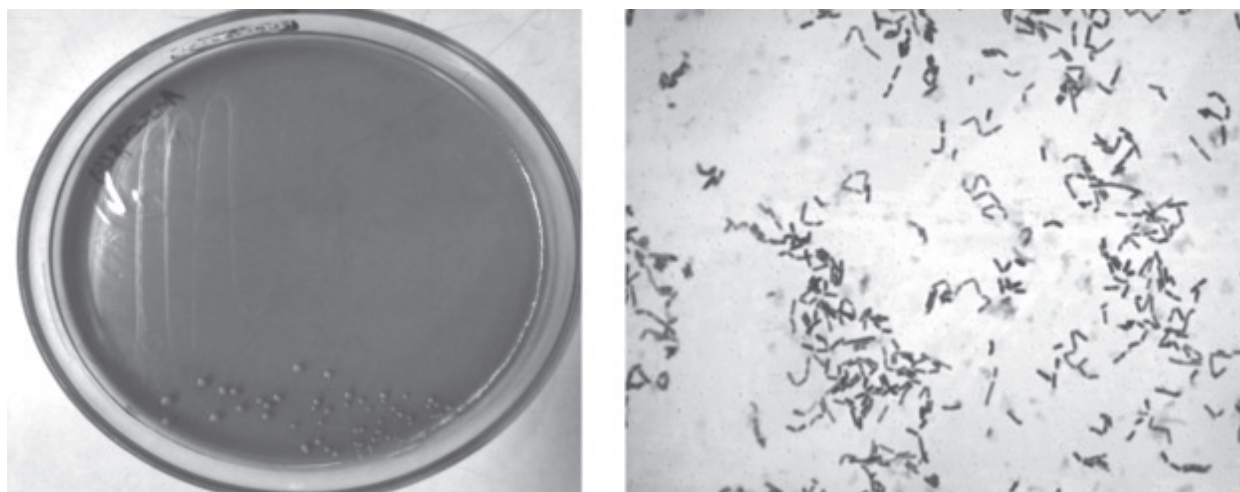

Figure 1. *Identification of Lactobacillus sp. based on (a) growth on MRS agar and (b) Gram staining*
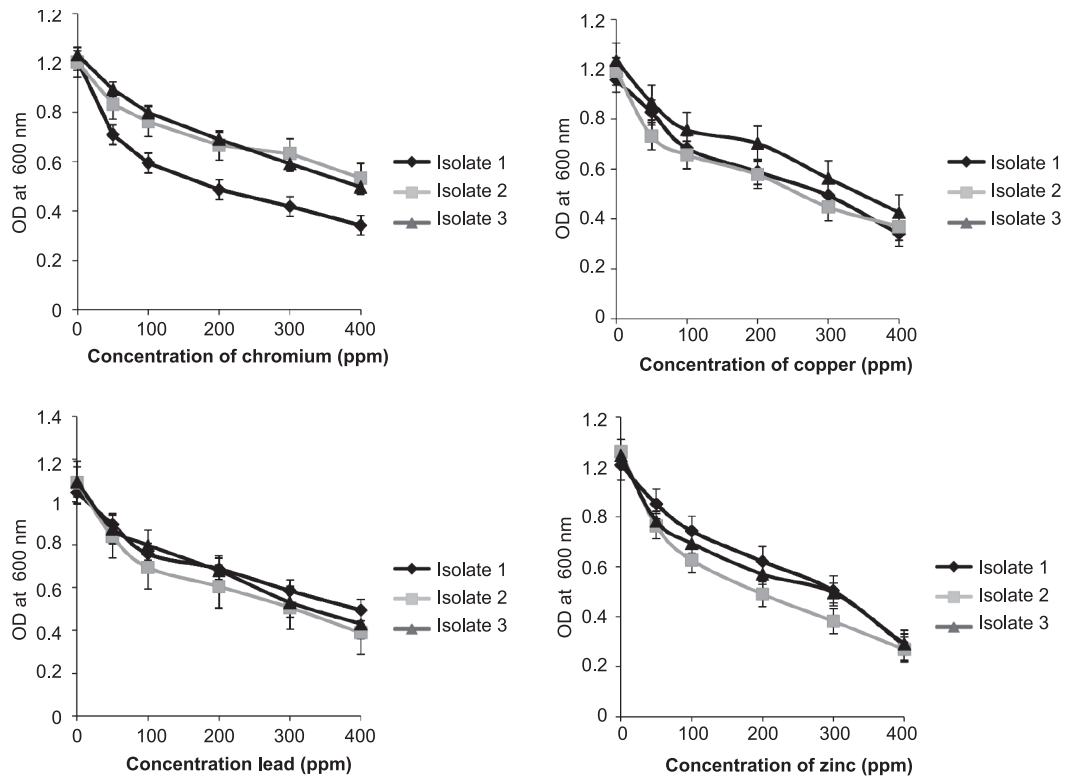
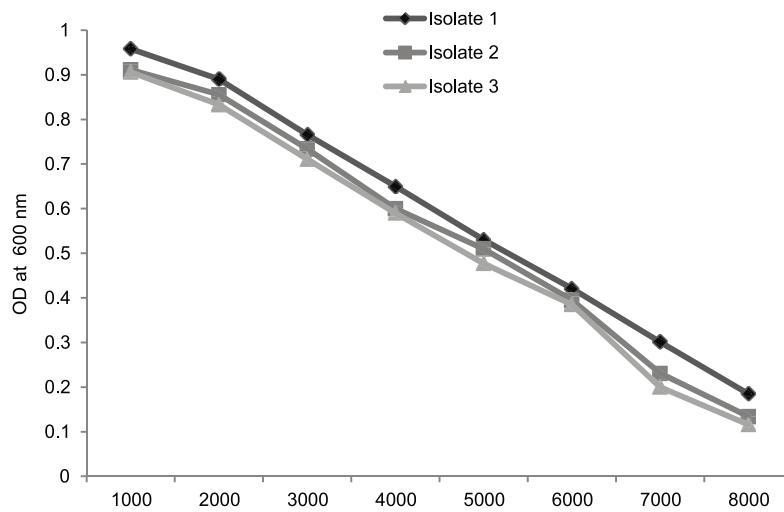**Figure 2.** *Tolerance of the Lactobacillus isolates to different heavy metals*

Figure 3. Bile salt tolerance of the *Lactobacillus* isolates

Table 1. *Acid tolerance of Lactobacillus isolates*

pH value	Growth of isolates		
	Isolate 1	Isolate 2	Isolate 3
1	-	-	-
1.5	+	-	-
2.0	++	++	+
2.5	+++	+++	+++
3.0	+	-	-

-: No growth, +: Slight growth; ++: Moderate growth; +++: Confluent growth

Table 2. *Antibiotic resistance pattern of the Lactobacillus isolates*

Antibiotics	Isolates		
	1	2	3
Azithromycin (15 μg)	Resistant	Resistant	Resistant
Chloramphenicol (30 μg)	Intermediate sensitive	Resistant	Intermediate sensitive
Cloxacillin (5 μg)	Resistant	Resistant	Resistant
Streptomycin (15 μg)	Resistant	Resistant	Resistant
Gentamycin (10 μg)	Resistant	Resistant	Resistant
Imipenem (10 μg)	Sensitive	Sensitive	Sensitive
Vancomycin (30 μg)	Resistant	Resistant	Resistant
Nalidixic acid (30 μg)	Resistant	Resistant	Resistant
Trimethoprim-sulfamethoxazole (25 μg)	Resistant	Resistant	Resistant
Penicillin–G (10 μg)	Resistant	Resistant	Resistant
Tetracycline (30 μg)	Resistant	Resistant	Intermediate sensitive

observations of multi-drug resistance among *Lactobacillus* spp. have been reported by other researchers[15, 16]. Resistance to the commonly used antibiotics suggests that these isolates would be able to withstand the high antibiotic concentrations occasionally observed in the environment and human intestinal tract. Additionally, ingestion of antibiotic often inhibits the residential microflora of the intestinal tract. Therefore administration of antibiotic resistant *Lactobacillus* will also help to maintain as well as restore the normal intestinal flora.

The *Lactobacillus* isolates obtained in this study are promising candidates for a safe and practical heavy metal bioremediation from environment and human body. Further investigation is needed to conduct in natural gastro-intestinal conditions to elucidate the exact removal capacity and mechanism. It is also necessary to study the heavy meal bioremediation capacity of these isolates in different physio-chemical conditions to determine the optimum parameters for environmental application.

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