

## Field studies on the removal of lead, cadmium and copper by the use of probiotic lactic acid bacteria from the water for culturing marine tilapia *T. spilurus*

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**Abstract:** The aim of this study was to examine the effect of probiotic lactic acid bacteria in removal lead, cadmium and copper from cultured water in fish farming system for marine tilapia *T. spilurus*, in addition studying the effect of heavy metals lead and cadmium and copper on genotoxicity of tilapia fish as bioindicator for heavy metal toxicity. A total number of 36 water samples from three localities of cultured fish farm; inlet of water, hatcheries (cement ponds) and rearing ponds (earthen ponds), The probiotic lactic acid bacteria (LAB) strains used in this study; *Lactobacillus rhamnosus* GG, *Lactobacillus fermentum* ME3, *Lactobacillus bulgaricus* (Commercial strain) and *Lactobacillus acidophilus* X 37 were used to remove lead, cadmium and copper from water of fish culture each sp. examined alone; the highest total concentration of removal was by *L. acidophilus* X37 (97.6) followed by *L. rhamnosus* GG (74.8), then *L. fermentum* ME3 (71.16) while the lowest concentration of removal was by *L. bulgaricus* (61.00). It was found that the optimal pH for *L. fermentum* ME3 was 6.0 for *L. rhamnosus* GG was 6, for *L. bulgaricus* was 5.0 and for *L. acidophilus* X37 was 6.0 While the impact of water temperature was clear that the percentage of removal of heavy metals depend on temperature where, all strains showing highest activity at temperature between 25 °C and 37°C then the activity was declined at 43 °C. There was significant increase in micronucleus (MN) frequencies in erythrocytes of tilapia sp. exposed to heavy metals in the fish farm in comparison to control.

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**Key words:** probiotic; lactic acid bacteria; marine tilapia (*T. spilurus*); Heavy metals; pH; temperature.; micronucleus

### Introduction:

The rapid development of industry has resulted significant quantities of heavy metals released increasing pollution, which is a significant environmental hazard for invertebrates, fish, and humans (Uluturhan and Kucuksezgin, 2007). Metals are discharged into rivers, agriculture drainage which can be strongly accumulated and biomagnified along water, sediment, and aquatic food chain, resulting in sub lethal effects or death in local fish populations (Awad, 2012). Suspended sediments adsorb pollutants from the water, thus lowering their concentration in the water column. Heavy metals are inert in the sediment environment and are often considered to be conservative pollutants (Olivares-Rieumont *et al.*, 2005) although they may be released into the water column in response to certain disturbances (Agarwal *et al.*, 2005). Removal of heavy metals from water can be achieved with precipitation, flocculation, ion exchange, and membrane filtration. These methods are sometimes expensive, not effective at low metal concentrations, and produce sludge to be disposed. Thus, safe novel treatments should be searched for future decontamination targets (Halttunen *et al.*, 2007). A vast array of biological materials, especially

bacteria, algae, yeasts and fungi have received increasing attention for heavy metal removal and recovery due to their good performance, low cost and large available quantities (Wang and Chen, 2008).

Use of inactivated microbial biomass as an adsorbent, biosorption, has been suggested as an effective and economical alternative for the removal of toxic metals from water, and the removal of a number of different minerals by varying micro-organisms have been studied (Davis *et al.*, 2003; Mehta and Gaur, 2005). It was established the capability of specific lactic acid bacteria to remove cadmium and lead from water (Halttunen *et al.*, 2003, 2007, 2008).

Heavy metals present in waters not only endanger survival and physiology of the aquatic organisms (Handy, 1994) but also induce genetic alterations (Fabacher *et al.*, 1991) which may lead to mutations (Maccubin *et al.*, 1991) and/or carcinogenesis (Folmar *et al.*, 1993). The effects of the mutations can either be silent throughout many generations or have a significant impact on the gene pool of a population. For this reason, there is increasing interest in the use of bioindicators to study the effects of aquatic pollutants at the genomic level (Russo *et al.*, 2004).

Among the techniques to detect genetic and genotoxic effects, the micronucleus (MN) test is often used since it allows for convenient and easy application, in particular in genotoxicologic studies with fish as the bioindicators. Thus the aim of this study was to examine the effect of probiotic lactic acid bacteria in removal lead, cadmium and copper from cultured water in fish farming system for marine tilapia *T. spilurus*, in addition, studying the effect of heavy metals lead, cadmium and copper on genotoxicity of marine tilapia fish as bioindicator for heavy metal toxicity.

#### Materials and methods:

##### Water samples:

Private marine tilapia *T. spilurus* farm, its water contains concentrations of heavy metals; lead, cadmium and copper released from adjacent metal factories.

A total number of 36 water samples from three localities of cultured fish farm; inlet of water, hatcheries (cement ponds) and rearing ponds (earthen ponds), samples were taken in glass flasks, 500 ml volume were equipped with a cork stopper and open hand prides under the water surface then equipped again and fixed with 200  $\mu$ l of pure concentrated nitric acid. Water samples were collected as five replicates from various depths along the three localities at the farm and the mean of their analysis were taken with standard deviation.

##### Bacterial strains:

The probiotic lactic acid bacteria (LAB) strains used in this study were *Lactobacillus rhamnosus* GG, *Lactobacillus fermentum* ME3, *Lactobacillus bulgaricus* (Commercial strain) and *Lactobacillus acidophilus* X 37 were kindly supplied from King Abd-El Aziz Medical City (National Guard Hospital) in Jeddah. Bacterial strains were examined each alone with specific pH and temperature. The study intended to use species of bacteria used as probiotic and immune stimulant for cultured fish.

##### Growth media and culture conditions:

Probiotic bacterial strains were cultured in MRS-broth for 48 h at 37°C. Biomass was then centrifuged (8000 $\times$ g, 15 min) and washed twice with ultra-pure water. Washed biomass was lyophilized and stored at -20 °C. till used in the experiment.

##### Binding assay:

Suspensions of lyophilized bacteria (2 g/l) were spiked with the water samples and diluted to a final bacterial concentration of 1 g/l and a final metal concentrations of (1.38 $\pm$ 0.069) mg/l, (2.13 $\pm$ 0.007) mg/l and (4.68 $\pm$ 0.057) mg/l for Pb<sup>2+</sup>, Cd<sup>2+</sup> and Cu<sup>+</sup> respectively. The pH of the metal solution was adjusted to 6 for *L. fermentum* ME3, for *L. rhamnosus* GG, and

for *L. acidophilus* X37 but for *L. bulgaricus* was 5 after experiments using dilute NaOH and HNO<sub>3</sub> and kept constant. Three 1.5 ml samples were taken from the suspension and these were incubated for 5h. at 37 °C. After incubation, the pH of the suspension was measured and bacteria were separated by centrifugation (7000  $\times$ g 5 min).

Supernatant was preserved with 1ml/ 200  $\mu$ l of pure conc. nitric acid and stored at room temperature. The effect of pH (1,2,3,4,5 and 6), and temperature (5,10,15, 25 and 37 °C) were tested, bacterial concentration used was 2.0 g for each litter. Conditions were selected, based on studies and experiments. All experiments were repeated at least twice (Halttunen *et al.*, 2007).

##### Analysis of cadmium, lead and copper:

Lead, cadmium and copper concentrations were determined with atomic absorption spectrometry either by flame or graphite furnace method depending on the metal concentration before and after insertion of lactic acid bacterial species. In each analysis, samples spiked with lead, cadmium and copper were used as quality control samples.

##### Data analysis:

Metal removal from cultured water was calculated firstly; by determining heavy metal concentration for each metal before and after using probiotic lactic acid bacteria for each species of bacteria alone.

Then by the equation described by Achanai Buasri *et al.*(2012). The amount of removed pb<sup>2+</sup>, Cd<sup>2+</sup> and Cu<sup>2+</sup> ions (mg metal ions/g biomass) were calculated from the decrease in the concentration of metal ions in the medium by considering the removed volume and used amount of the lactic acid bacteria:

$$q_e = (C_i - C_e) V / m$$

where  $q_e$  is the amount of metal ions removed into unit mass of the biosorbent (mg/g) at equilibrium,  $C_i$  and  $C_e$  are the initial and final (equilibrium) concentrations of the metal ions in the solution (ppm),  $V$  is the volume of metal solution (L) and  $m$  is the amount of lyophilized lactic acid bacteria used (g).

##### Preparation of lactic acid bacteria for scanning electron microscopy:

Bacteria were grown in aerated liquid media to the exponential phase and harvested by centrifuging at 1000 g for 30 min. The bacterial pellet was fixed with 1 % (v/v) glutaraldehyde for 30 min then with 1 % (w/v) osmium tetroxide for 12 to 20 h, both treatments being done in a refrigerator. Fixatives were prepared in 0.15 M-sodium cacodylate buffer, pH 7.0. The fixed pellet was dehydrated in a graded ethanol series from 50 % to 100 %, followed by amyl acetate, then dried by the 'critical point' method. The dried pellet was broken into small fragments with the tip of forceps and

pressed on to an aluminium block. The preparation was then coated with gold/palladium (60: 40). The thickness of the metal coating was controlled so that it did not exceed 10 nm, using a deposit thickness monitor (DTM-2; Sloan Instrument Corp., Santa Barbara, California, U.S.A.). Bacteria was examined using a scanning electron microscope (JEOL SEM T330; JOEL, Japan) operating at 25 kV. **Amako and Umeda (1977).**

#### Micronuclei assay:

Five fish from the rearing ponds were used for micronuclei assay. Peripheral blood samples were obtained from the caudal vein of the tilapia fish and smeared onto pre-cleaned slides. After fixation in pure ethanol for 20 min, the slides were allowed to air-dry and then the smears were stained with Giemsa. From each fish three slides were prepared, and from each slide 1500 polychromatic erythrocytes were scored under 100 x magnifications. Frequencies of micronuclei was compared with the frequencies of micronuclei in erythrocytes of tilapia fish from another farm (control). Small, non-refractive, circular, or ovoid chromatin bodies, displaying the same staining as the main nucleus, were scored as micronuclei (**Al-Sabti and Metcalfe, 1995**).

#### Results:

#### Results of removal of heavy metals at rearing ponds by probiotic lactic acid bacteria:

Table 1. Percentage of removal of heavy metal ions ( $\mu\text{g/l}$ ) onto the surface of probiotic lactic acid bacteria

Lactic acid bacteria	Heavy metal conc ( $\mu\text{g/l}$ )			Heavy metal conc removed ( $\mu\text{g/l}$ )						Total %
	Pb	Cd	Cu	Pb removed	% of removal	Cd removed	% of removal	Cu removed	% of removal	
L. fermentum ME3	1.38 ± 0.069	2.13 ± 0.007	4.68 ± 0.057	0.92 ± 0.184	66.6	1.56 ± 0.312	73.2	3.45 ± 0.690	73.7	71.16
L. rhamnosus GG	1.77 ± 0.089	2.73 ± 0.107	3.97 ± 0.980	0.89 ± 0.222	50.2	2.20 ± 0.440	80.5	3.73 ± 0.927	93.9	74.8
L. bulgaricus *(Co. st.)	1.34 ± 0.117	2.22 ± 0.014	3.75 ± 0.870	0.45 ± 0.080	33.5	1.10 ± 0.220	49.5	3.75 ± 0.770	100	61.0
L. acidophilus X37	1.12 ± 0.050	2.17 ± 0.024	4.34 ± 0.970	1.04 ± 0.208	92.8	2.17 ± 0.542	100	4.34 ± 0.868	100	97.6

Heavy metal conc. (mean of three replicates) ± standard deviation

\* Commercial strain

#### Factors affecting concentration of heavy metal removed from water

Experiments was done to examine the various factors affecting the concentration of removing heavy

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With determining the concentration of removal of mixed heavy metals ( $\text{Pb}^{+2}$ ,  $\text{Cd}^{+2}$  and  $\text{Cu}^{+2}$ ) from cultured water at the fish farm using investigated strains of probiotic lactic acid bacteria each alone, it was clear from the Fig 1 and table 1 that the highest total concentration of removal was by *L. acidophilus* X37 (97.6) followed by *L. rhamnosus* GG (74.8), then *L. fermentum* ME3 (71.16) while the lowest concentration of removal was by *L. bulgaricus* (61.00).

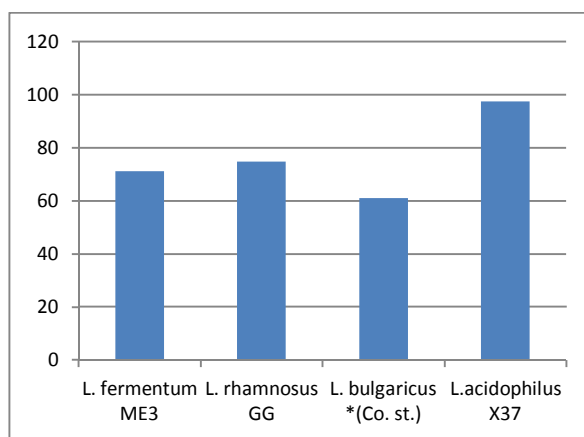


Figure 1. percentage of removal of heavy Metals by lactic acid bacteria each alone

metals from cultured water determining the optimal factors; cadmium, lead and copper removal assessment indicated a strongly pH-dependent process with the highest removal at a pH close to neutral (Fig.2).

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it was found that the optimal pH for *L. fermentum* ME3 was 6, for *L. rhamnosus* GG was 6, for *L. bulgaricus* was 5 and for *L. acidophilus* X37 was 6. While the impact of water temperature was clear that the percentage of removal of heavy metals depend on temperature where, all strains showing highest activity at

temperature between 25 °C and 37°C then the activity was declined at 43 °C (Fig.3). There is leaner relation between the concentration of lactic acid bacteria and percentage of heavy metal removal.

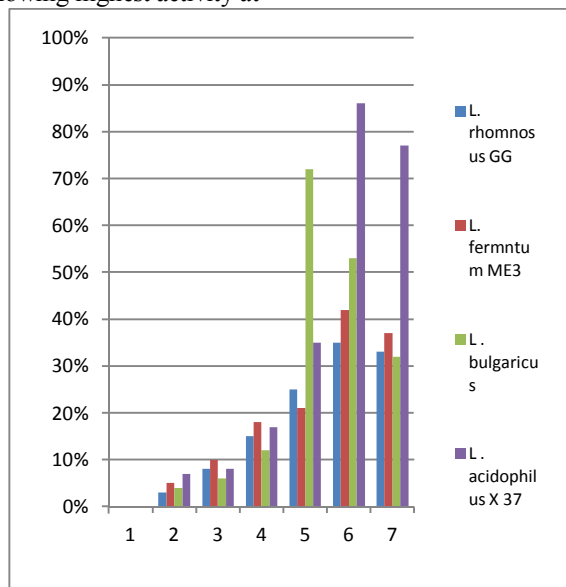


Figure 2. pH of water and percentage of removal of heavy metal from water

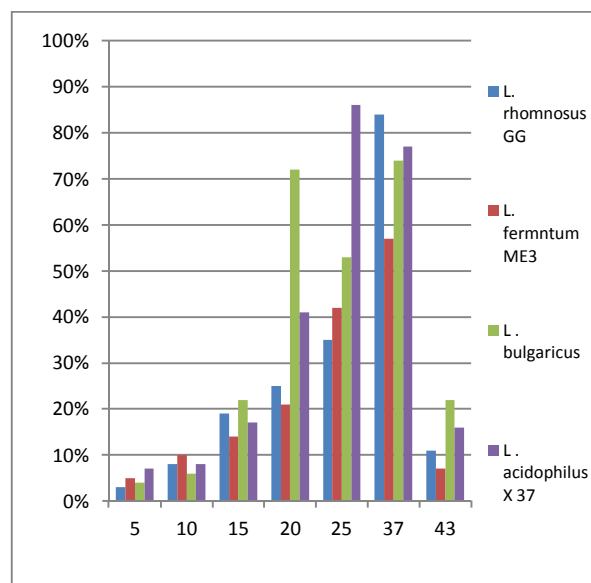


Figure 3. Temperature of water and percentage of removal of heavy metal from water

#### Results of scan electron microscope:

Scan electron microscope was performed for *L. acidophilus* X37 before and after spiked with polluted

water with lead, cadmium and copper for confirmation, Scan electron micrograph figure 4 (A&B).

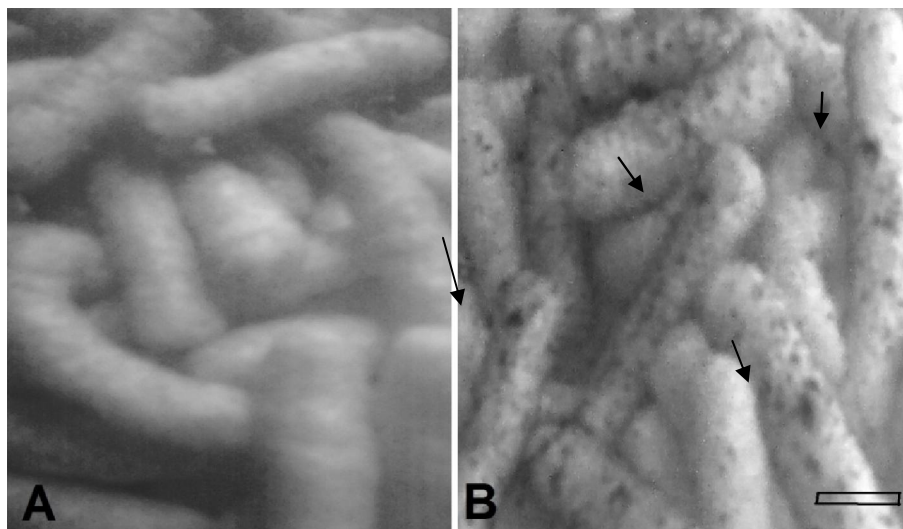


Figure 4. (A) Scanning electron micrograph for pellets of *L. acidophilus* X37 (LAB) before spiked with contaminated water with heavy metals, (B) *L. acidophilus* X37 adsorb mixed heavy metals (arrows) from water of fish culture. (scale bar represent 100 nm.)

#### Results of micronucleus assay:

There was significant increase in MN frequencies in erythrocytes of tilapia sp. exposed to

heavy metals in the fish farm in comparison to control (tilapia fish from another farm) fig (4).

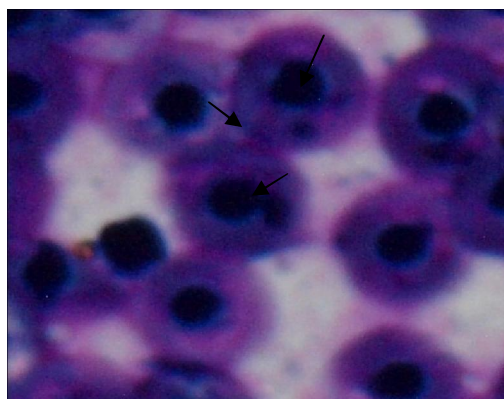


Figure 5. Showing RBCs of *Tilapia spirulus* stained with Giemsa exposed to pollution with mixed heavy metals showing micronucleus (arrows)

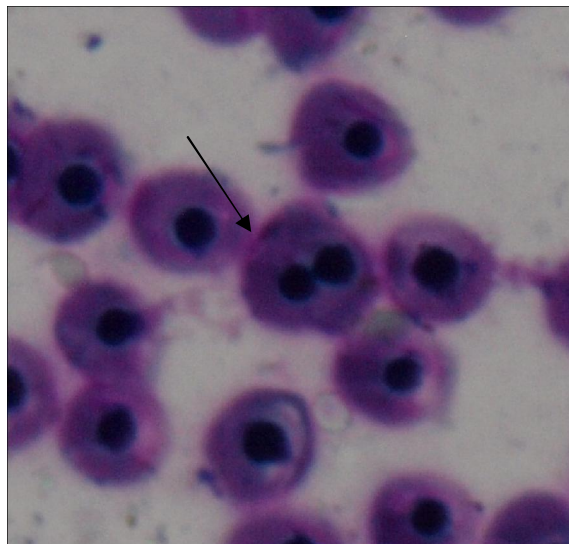


Figure 6. Showing RBCs of *Tilapia spirulus* stained with Giemsa exposed to pollution with mixed heavy metals showing double-nucleated RBC (arrow)

#### Discussion:

The goal of this study was to assess and performed in the field in tilapia (*T. spirulus*) fish farm naturally contaminated with mixed heavy metals lead, cadmium and copper so as to workout lactic acid bacteria which is also used as probiotic immunostimulant and growth promoter for fish in fish farm and different study from its predecessor that most of previous studies were done in the laboratory or been tested in the laboratory and not in the field.

Heavy metal contamination may have devastating effects on the ecological balance of the recipient environment and a diversity of aquatic organisms (Ayandiran *et al.*, 2009). They can decline water and sediments quality and may adversely affect fish health and other biological attributes like taxonomic richness, trophic structure, and health of individual organisms (Batziar and Siontoro, 2008). They can also form a major hazard because of their toxicity, persistence, and bioaccumulation in the food chains (Djedjibegovic *et al.*, 2012). Removal of heavy metals from water can be achieved with precipitation, flocculation, ion exchange, and membrane filtration. These methods are sometimes expensive, not effective at low metal concentrations, and produce sludge to be disposed. Thus, safe novel treatments should be searched for future decontamination targets (Halttunen *et al.*, 2007). Probiotic bacteria have the capacity to bind many toxic compounds like aflatoxins (Peltonen *et al.*, 2001; Haskard *et al.*, 2001), food-borne mutagens (Turbic *et al.*, 2002) and microcystin-LR (Meriluoto *et al.*, 2005) from aqueous solution. There is also some evidence that probiotic bacteria could bind aflatoxin B1 (El-Nezami *et al.*, 2000, 2006) and the food-borne mutagen Trp-P-2 (Orrhage

*et al.*, 2002) within the gastro-intestinal tract, thereby reducing their uptake (Halttunen *et al.*, 2007). The potential of different microbes in removal of heavy metals and other toxic compounds from water has been recently recognized. It was reported that lactic acid bacteria (LAB) effectively remove cationic heavy metals, cadmium and lead (Ibrahim *et al.*, 2006; Halttunen *et al.*, 2007), from water.

Present study revealed that 4 species of probiotic lactic acid bacteria; *Lactobacillus rhamnosus* GG (ATCC 53103), *Lactobacillus fermentum* ME3, *Lactobacillus bulgaricus* (Commercial strain) and *Lactobacillus acidophilus* X 37 have remove heavy metals; lead, Cadmium and copper from water at fish culture with different degrees and forces with different species used. Cadmium and lead removal was observed to occur rapidly to bacterial surface, probably by an ion exchange mechanism as LAB have negative surface charge, they are optimal for cation binding the results nearly agree with the results obtained by Halttunen *et al.* (2007). Present study indicate that probiotic lactic acid bacteria have potential to remove mixed concentrations of heavy metals lead, cadmium and copper from water with different degrees also (Sheng *et al.*, 2007) observed that from binary metal solutions containing lead and cadmium, zinc, nickel, cobalt, potassium, sodium, calcium or magnesium. Many reasons have been suggested for selectivity of some metals over others. Many literatures indicates that the removal occurs at the bacterial surface probably by an ion exchange mechanism, these studies confirm the results of present study. Lactic acid bacteria have also been reported to remove also mycotoxins (Pierides *et al.*, 2000) and cyanotoxins (Meriluoto *et al.*, 2005; Nybom *et al.*, 2007) from food and water.

Removal of heavy metal ions onto the surface of a probiotic lactic acid bacteria is affected by several factors, such as initial solution concentration, initial biomass concentration, temperature and pH of solutions, present study indicate that cadmium, lead and copper removal assessment indicated a strongly pH-dependent process with the highest removal at a pH close to neutral. It was found that the optimal pH for *L. fermentum* ME3 was 6, for *L. rhamnosus* GG was 6, for *L. bulgaricus* was 5 and for *L. acidophilus* X37 was 6. While the impact of water temperature was clear that the percentage of removal of heavy metals depend on temperature where, all strains showing highest activity at temperature between 25°C and 37°C then the activity was declined at 43°C.

Many factors affect the growth and activity of bacteria: temperature, pH, oxygen, salt concentration and nutrients are some of the more common factors that may change in the normal environment of bacteria. While most bacteria grow best when these parameters are optimum for that strain. The pH of the environment affects bacterial growth. Most bacteria grow best in the pH range from about 6-8; however, there are many acid-tolerant bacteria as well as alkaline-tolerant strains. In general, bacteria survive alkaline pH better than acid pH, but a few strains actually grow better in an acidic environment. Some can even use sulfuric acid as an energy source. The pH of the cell contents of bacteria that grow in acidic or alkaline environments is neutral. These strains have transport mechanisms to keep a normal physiological H<sup>+</sup> ion concentration inside the cell so the activity of used probiotic lactic acid bacteria mainly pH- dependent (**Sathe et al., 2007**).

The temperature in many natural environments changes drastically over the seasons. While most of the well-characterized bacteria live best at temperatures from 25°-40°C, many bacteria thrive at high temperatures and others grow best (although slowly) at 0°-15° C. Every organism has an optimum temperature for growth and activity. It was clear that the used probiotic lactic acid bacteria increasingly activated at 25 °C – 37 °C (**Reddy and Ranganathan 1985; Batish et al., 1997; Zaitseva et al., 2004**).

Regarding the results of scan electron microscope, Although the images of electron microscope for lactic acid bacteria were not good for technical reasons confirmed that the lactic acid bacteria is the main reason for the removal of heavy metals from water of aquaculture. SEM was performed for *L. acidophilus* X37 before and after spiked with polluted water with lead, cadmium and copper for confirmation, that the removal of heavy metals was due to probiotic lactic acid bacteria (LAB). The results nearly agree with the results obtained by **Halttunen et**

**al.(2008)** who mentioned that the transmission electron microscope when performed for lyophilized *B. longum* 46 and *L. fermentum* ME3 before and after lead binding showed as large deposits of lead on the surface of both strains after binding.

Regarding genotoxic studies micronucleus assay from the present study, MN assay is a significant and potent tool for the assessment of genotoxicity as it is simple, reliable, sensitive, and is dependent of karyotypic characteristics of the test animal. Fish are excellent specimens for the study of the mutagenic or carcinogenic potential of contaminants present in water sample since they can metabolize, concentrate and store waterborne pollutants **Yadav and Trivedi (2009)**. They can serve as useful genetic models for the evaluation of pollution in aquatic ecosystems (**Park et al.,1993**). The erythrocyte micronucleus test has been used with different fish species to monitor aquatic pollutants displaying mutagenic features (**Pantalea et al.,2006**). **Rodriguez et al.,(2003)** have also reported the sensitivity of micronucleus test in fish species for application in field surveys. In present study that there were significant difference in frequency in erythrocytes of tilapia exposed to heavy metals; lead, cadmium and copper in comparison with control the results nearly agree with the results obtained by **Galan et al.,(2001)** who reported that there is significant increase in frequency of micronucleus was found in eels injected with cadmium or mercury. **Manna and Sadhukon (1986)** also reported that cadmium also induces micronuclei formation in tilapia fish. From the investigation it was concluded that probiotic lactic acid bacteria have been identified as safe potent tool for the decontamination of water of fish cultures from heavy metals; lead, cadmium and copper with different degrees according to species of lactic acid bacteria and kind of heavy metal removed.

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