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In vitro development of resistance to arsenite and chromium-VI in Lactobacilli strains as perspective attenuation of gastrointestinal disorder

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Abstract

Inadvertent intake of inorganic arsenic and chromium through drinking water and food causing their toxic insults is a major health problem. Intestinal bacteria including Lactobacilli play important regulatory roles on intestinal homeostasis, and their loss is known to cause gastrointestinal (GI) disorders. Probiotic Lactobacilli resistance to arsenite and chromium-VI could be an important factor for the perspective attenuation of GI-disorders caused by these toxic metals/metalloid. In the present study resistance of arsenite (up to 32 ppm), Cr-VI (up to 64 ppm), and arsenite plus Cr-VI (32 ppm each) were developed under *in vitro* condition following chronological chronic exposures in Lactobacilli strains. Comparative study of biochemical parameters such as membrane transport enzymes and structural constituents; dehydrogenase and esterase activity tests, which are respective indicators for respiratory and energy producing processes, and the general heterotrophic activity of cells, of resistant strains showed similarities with their respective normal parent strains. The resistant strains were also found to be sensitive to antibiotics. Findings indicate that these resistant probiotic Lactobacilli would be useful in the prophylactic interventions of arsenic and chromium GI-toxicity.

Key words

Arsenic, Chromium, GI toxicity, Lactobacilli, *In vitro* resistance, Probiotics

Introduction

Arsenic and chromium ingestion through drinking water and food causing their toxic insults leading to severe diseases including gastrointestinal (GI) disorders are common environmental pollution related problems in several countries. Ingestion of arsenic and chromium is known to cause irritation of the digestive tract leading to GI-disorders including pain, nausea, vomiting and diarrhea (Toxicological Profile for Chromium, 2000; Arsenic, 2005). It is difficult to remove these toxic metals/metalloids from food and water sources. The intestinal epithelium is the first physiological barrier to arsenic and chromium metabolism and distribution towards the tissues through the blood stream. Mammalian intestinal epithelial cells function while in physical contact with an ecosystem of bacteria and maintain a dynamic interrelationship. It is well established that the intestinal bacteria has regulatory effect on intestinal homeostasis (Bengmark, 1998; Hooper *et al.,* 2001). It is also known that a breakdown in the relationship between intestinal epithelial cells and bacteria results in the manifestation of GI-disorders (Neish, 2002; Hart *et al.,* 2002). This has been seen in case of oral exposure of arsenic and chromium. Probiotic bacteria favorably alter the intestinal microflora balance and Lactobacilli belong to the microorganisms most frequently used as probiotics. They have the ability to adhere to the epithelial cells of the gut, genetically stable and have good growth properties *in vitro* and *in vivo* and are able to maintain its

high viability at processing, lyophilization and storage (Kirjavainen *et al.,* 1998; Morelli, 2000; Marteau *et al.,* 2001). Multiple potential beneficial effects have been attributed to the probiotics use of Lactobacilli in a number of infective and noninfective disorders (Shanahan, 2004; Sharma *et al.,* 2005). However, insufficient functions of Lactobacilli, if used as probiotics, due to the problem of their survival in case of continued exposure of Arsenic and/or Chromium through drinking water and food, may not provide long term fruitful advantages. On the other hand, the use of As/Cr-resistant Lactobacilli may provide better health benefits against As/Cr-toxicity. Microorganisms have evolved resistance mechanism to deal with metal toxicity as the result of exposure to metal contaminated environments. Some bacteria present in water and soil develop resistance to chromium on exposure to Cr-containing effluent in their environment. These bacteria reduce Cr-VI into Cr-III and minimize the adverse effects of Cr-VI on their growth. In addition, it has also been reported that the different cells in the body differ vastly in their capacity to reduce Cr-VI, the most efficient being the intestine due to the presence of cells like epithelial cells and bacterial cells (Shrivastava *et al.,* 2003). Similarly, due to the abundance of arsenic at near toxic levels in the environment since the origin of life, microbes have evolved mechanisms for arsenic resistance. Therefore, in the present study attempts have been made to develop Arsenite, Cr-VI and Arsenite plus Cr-VI-resistance in Lactobacilli strains that are commonly found in mammalian GI-tract, under *in vitro* condition. Furthermore, to ascertain the similarities amongst the respective parent strain and the resistant strain, growth phase and various biochemical parameters have been compared.

Materials and Methods

Animals and chemicals: Sodium m-arsenite was purchased from Sigma-Aldrich and potassium dichromate was from SISCO Research Laboratory (SRL) India. All other chemicals, reagents and media were purchased either from Sigma-Aldrich, E. Merck, Gibco, SRL, HiMedia, India. Healthy adult albino Wistar rats procured from the Animal Breeding Facility of Indian Institute of Toxicology Research, Lucknow, were used for the isolation of intestinal bacteria and for *in situ* studies. Clearance from the Animal Ethical Committee of the Institute was obtained for the use of animals.

Organism: Pure cultures of Lactobacilli strains *viz*. *L. acidophilus* (MTCC 447), *L. rhamnosus* (MTCC 1408) and *L. casei* (MTCC 1423) were procured from the Institute of Microbial Technology (IMTECH), Chandigarh, India and maintained in the laboratory. *L. acidophilus* and *L. rhamnosus* were cultivated in MRS broth and *L. casei* in Tomato juice broth (HiMedia).

MIC determination: The minimal inhibitory concentration (MIC) of arsenite (As-III) and chromium-VI (Cr-VI) was determined by the standard broth agar dilution method. Viability was tested by CFU on agar plates.

Serial passages: Parent strains were fully grown in respective media at 37°C for 18 hr, and approximately 5 x 10^7 CFU of each strain were inoculated into each of a series of tubes containing 9.9 ml of appropriate broth with arsenite or chromium-VI or arsenite plus Cr-VI concentrations consisting of doubling dilutions below and above the MIC. Following the incubation at 37°C for 24 hr, aliquots from the tube nearest the MIC, which had the same turbidity as the arsenite/Cr-VI free control, were used following a 1:100 dilution to inoculate a second set of tubes containing the appropriate broth with arsenite/Cr-VI. After overnight incubation, the bacteria were transferred again and 10-12 serial transfers were carried out. Likewise, each strain was then cultivated in doubling concentrations. The glycerol stock of resistant strains were kept frozen at – 40°C and time to time sub cultured to assess the stability of resistance.

Growth phase studies: An approximately equal number of cells (2 x 10^6 cells) of normal parent strains and the respective arsenite, Cr-VI, arsenite plus Cr-VI-resistance developed strains of Lactobacilli were grown in desired media at 37°C. Bacterial growth was measured at different time intervals up to 30 hr using turbidimetry at 610 nm. In case of resistant strains, growth measurements were carried out in the absence and presence of respective concentrations of arsenite, Cr-VI, and arsenite plus Cr-VI in the media. The specific growth rate, doubling time and number of generations of bacteria were evaluated as described by Espigares and Mariscan (1989).

Antibiotic sensitivity by disk diffusion: All the normal parent strains and respective arsenite, Cr-VI and arsenite plus Cr-VI-resistance developed strains of Lactobacilli were tested for antibiotic sensitivity following the National Committee for Clinical Laboratory (NCCL) standard disk diffusion method. The following antibiotic disks from HiMedia, India were used: Amoxycillin (25 µg), Chloramphenicol (25 µg), Ciprofloxacin (10 µg), Erythromycin (10 µg), Gentamycin (10 µg), Kanamycin (30 µg), Norfloxacin (10 µg), Novobiocin (30 µg), Streptomycin (10 µg) and Teicoplanin (30 µg).

***In situ* studies:** Rat intestinal loop studies were carried out as described by Upreti *et al.* (2008). Intestinal loops were filled with different concentrations of arsenite, Cr-VI or arsenite plus Cr-VI and incubated for 30 min under *in situ* conditions. Intestinal epithelial cell (IEC) membrane was prepared as per Upreti *et al.* (2005).

Enzyme assays and biochemical estimations: Dehydrogenase (DHA) and esterase activity (EA) tests were carried out as described by Liu (1985) and Obst and Holzapfel-Pschorn (1988), respectively. Bacterial cell membrane was prepared as described by Kumar and Upreti (2000). Alkaline phosphatase (EC 3.1.3.1) was determined according to Weiser (1973) and Ca^{2+}-Mg^{2+}-ATPase (EC 3.6.1.3) as described by Hidalgo *et al.* (1983). Enzyme units were defined as micromoles of product formed or liberated per minute under the assay conditions. Specific activity was expressed as units per milligram of protein. Protein was determined according to Lowry *et al.* (1951) using bovine serum albumin as standard. Carbohydrate (total hexose) and sialic acid were respectively estimated according to Monsigny *et al.* (1988) and Warren (1959). Total lipid was extracted according to Folch *et al.* (1957). Phospholipids were quantified following digestion with 70% perchloric acid and estimated according to the method of Wagner *et al.* (1962).

Table - 1: Specific growth rate, doubling time and number of generations of normal, and arsenite, Cr-VI, and arsenite plus Cr-VI-resistant Lactobacilli strains.

Lactobacillus strains	Arsenite-resistant			Cr-VI-resistant			Arsenite + Cr-VI-resistant		
	6 hr	12 hr	24 hr	6 hr	12 hr	24 hr	6 hr	12 hr	24 hr
Sp. growth rate									
L. acidophilus-Normal	0.66	0.38	0.20	0.72	0.39	0.20	0.62	0.39	0.20
L. acidophilus-Resistant	0.58	0.37	0.19	0.48	0.36	0.19	0.62	0.38	0.19
L.casei-Normal	0.76	0.41	0.22	0.59	0.36	0.19	0.70	0.40	0.20
L.casei-Resistant	0.67	0.41	0.21	0.59	0.37	0.20	0.51	0.41	0.21
L. rhamnosus-Normal	0.71	0.40	0.20	0.55	0.40	0.20	0.59	0.39	0.19
L.rhamnosus-Resistant	0.38	0.30	0.20	0.37	0.35	0.19	0.54	0.36	0.20
Doubling time (min.)									
L. acidophilus-Normal	62.8	109.2	213.2	57.5	125.7	210.5	67.2	125.2	207.0
L. acidophilus-Resistant	71.9	112.4	214.2	87.2	136.9	217.9	92.1	129.1	208.8
L.casei-Normal	54.9	101.4	192.3	70.7	133.5	214.6	59.7	120.8	207.0
L.casei-Resistant	61.7	102.7	196.8	70.1	132.0	210.1	81.8	117.6	196.8
L. rhamnosus-Normal	58.7	103.7	203.7	75.5	122.6	204.9	70.7	125.7	213.2
L.rhamnosus-Resistant	108.5	140.6	211.4	111.4	137.6	210.5	77.5	134.8	211.9
No. of generations									
L. acidophilus-Normal	5.7	6.6	6.8	6.3	6.7	6.8	5.4	6.7	7.0
L. acidophilus-Resistant	5.0	6.4	6.7	4.1	6.1	6.6	5.4	6.5	6.9
L.casei-Normal	6.6	7.1	7.5	5.1	6.3	6.7	6.0	7.0	7.0
L.casei-Resistant	5.8	7.0	7.3	5.1	6.4	6.9	4.4	7.1	7.3
L. rhamnosus-Normal	6.1	6.9	7.1	4.8	6.9	7.0	5.1	6.7	6.8
L.rhamnosus-Resistant	3.3	5.1	6.8	3.2	5.6	6.8	4.7	6.2	6.8

Values are mean of five to seven replicate samples. Variance was within a limit of 10-15%.

Table - 2: Membrane enzymes of normal and arsenite, Cr-VI and arsenite plus Cr-VI- resistant (32 ppm each) Lactobacilli strains

Lactobacillus strain	Specific activity (mg^{-1} protein)	
	Alkaline phosphatase	Ca^{2+}- Mg^{2+} - ATPase
Arsenite-resistant		
L. acidophilus - Normal	1.92 ± 0.20	0.196 ± 0.022
L. acidophilus – Resistant	1.93 ± 0.22	0.185 ± 0.019
L. casei – Normal	1.56 ± 0.16	0.121 ± 0.013
L. casei – Resistant	1.36 ± 0.15	0.108 ± 0.011
L. rhamnosus – Normal	1.71 ± 0.18	0.147 ± 0.016
L. rhamnosus – Resistant	1.61 ± 0.17	0.131 ± 0.014
Cr-VI-resistant		
L. acidophilus - Normal	1.44 ± 0.15	0.168 ± 0.018
L. acidophilus – Resistant	1.28 ± 0.14	0.155 ± 0.017
L. casei – Normal	1.39 ± 0.15	0.152 ± 0.016
L. casei – Resistant	1.42 ± 0.15	0.160 ± 0.018
L. rhamnosus – Normal	1.73 ± 0.18	0.125 ± 0.014
L. rhamnosus – Resistant	1.42 ± 0.11	0.117 ± 0.012
Arsenite + Cr-VI-resistant		
L. acidophilus - Normal	1.51 ± 0.15	0.199 ± 0.021
L. acidophilus – Resistant	1.39 ± 0.15	0.177 ± 0.019
L. casei – Normal	1.43 ± 0.15	0.168 ± 0.017
L. casei – Resistant	1.33 ± 0.14	0.163 ± 0.017
L. rhamnosus – Normal	1.65 ± 0.17	0.101 ± 0.011
L. rhamnosus – Resistant	1.303 ± 0.10*	0.090 ± 0.010

Values are mean of three replicates ± SD, *p<0.05

Statistical analysis: The results are expressed as mean±SD and comparisons were made with appropriate controls using Student's t-test. Probability values of < 0.05 were considered to be significant.

Results and Discussion

Arsenite, Cr-VI and arsenite plus Cr-VI- resistance were developed in three Lactobacilli strains under *in vitro* conditions following chronological chronic exposure individually as well as in combination. Sub-culturing in the presence of arsenite, Cr-VI and arsenite plus Cr-VI gave increased MICs for all Lactobacilli strains, with MICs rising from 0.05 - 1.0 to 2.0 - 32 mg l^{-1} after 6 to 12 sub-cultures in arsenite; from 2.0 - 8.0 to 8.0 - 64 mg l^{-1} after 6 to 12 sub-cultures in Cr-VI and from 0.05 -1.0 to 2 - 32 mg l^{-1} after 8 to 15 sub-cultures in arsenite plus Cr-VI.

Growth profile of the bacteria: The comparative growth profiles of Lactobacilli strains of normal parent bacteria and respective arsenite, Cr-VI and arsenite plus Cr-VI-resistant strains are summarized in Fig. 1, 2. In general, arsenite, Cr-VI and arsenite plus Cr-VI-resistant bacteria when grown in presence of respective arsenite (32 ppm), Cr-VI (32 ppm) or arsenite plus Cr-VI (32 ppm each) concentrations in the media showed more or less similar growth pattern as that of their corresponding normal parent strains. However, in case of arsenite-resistant *L. rhamnosus*, a prolonged lag and early log phase along with an enhancement in doubling time as well as decrease in the number of generations were observed. After 14 hr the specific growth rate, doubling time and number of generations followed the similar pattern as that of normal parent bacteria (Table 1). In case of Cr-VI-resistant *L. acidophilus*

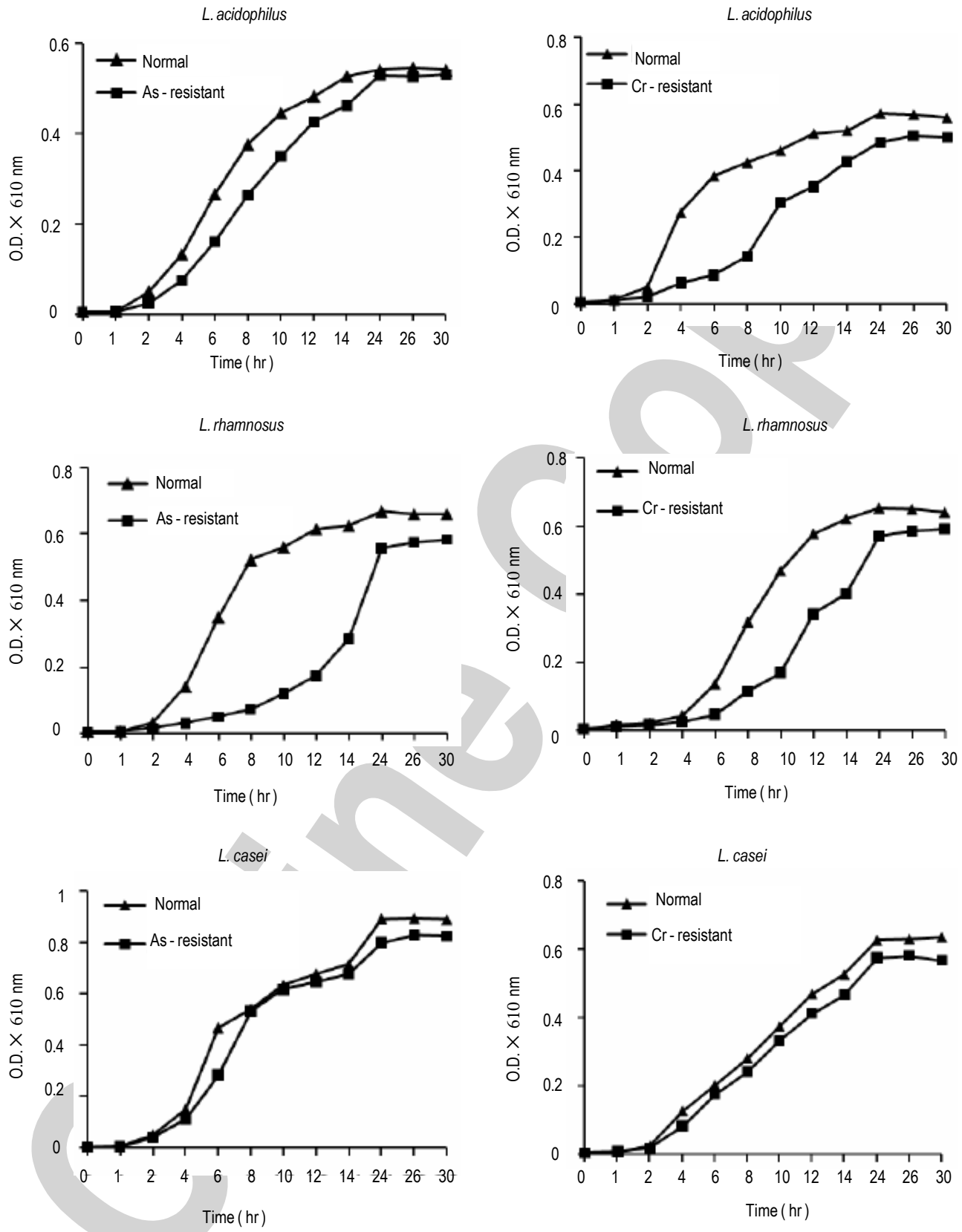

Fig. 1: Growth curves of normal, 32 ppm arsenite-resistant (As-resistant) and 32 ppm Cr-VI-resistant (Cr-resistant) *L.acidophilus*, *L.rhamnosus* and *L.casei*. Data represents mean value of five or seven replicates. SD has not been shown to avoid overcrowding. Variance was within a limit of 10-15%

Table - 3: Membrane constituents of normal, and arsenite, Cr-VI, and arsenite plus Cr-VI- resistant (32 ppm each) Lactobacilli strains

Lactobacillus strains	μg mg^{-1} protein		
	Hexose	Sialic acid	Phospholipids
Arsenite-resistant			
L. acidophilus – Normal	199.2 ± 21.5	19.4 ± 2.1	42.3 ± 4.4
L. acidophilus – Resistant	209.1 ± 22.2	16.2 ± 1.9	41.3 ± 4.3
L. casei – Normal	190.0 ± 20.4	20.6 ± 2.2	36.4 ± 3.8
L. casei – Resistant	210.3 ± 24.2	20.4 ± 2.4	37.7 ± 3.9
L. rhamnosus – Normal	200.1 ± 24.0	19.3 ± 2.1	38.9 ± 4.1
L. rhamnosus – Resistant	218.0 ± 25.5	18.0 ± 1.9	42.7 ± 4.4
Cr-VI-resistant			
L. acidophilus – Normal	178.4 ± 19.2	18.3 ± 2.0	23.5 ± 2.6
L. acidophilus – Resistant	170.0 ± 20.1	15.4 ± 1.4	26.0 ± 3.0
L. casei – Normal	186.5 ± 21.2	19.6 ± 2.1	21.8 ± 2.2
L. casei – Resistant	179.8 ± 19.6	15.5 ± 1.6*	23.0 ± 2.3
L. rhamnosus – Normal	193.6 ± 21.4	17.5 ± 1.8	24.4 ± 2.6
L. rhamnosus – Resistant	141.3 ± 14.0*	16.9 ± 1.8	30.2 ± 3.2
Arsenite + Cr-VI-resistant			
L. acidophilus – Normal	199.2 ± 21.2	19.3 ± 2.0	29.6 ± 3.1
L. acidophilus – Resistant	153.4 ± 15.8*	14.0 ± 1.4*	26.2 ± 2.8
L. casei – Normal	198.0 ± 21.5	21.1 ± 2.2	27.6 ± 2.9
L. casei – Resistant	170.3 ± 18.0	16.9 ± 1.8*	25.3 ± 3.0
L. rhamnosus – Normal	237.1 ± 24.8	19.3 ± 2.1	29.3 ± 3.0
L. rhamnosus – Resistant	180.1 ± 18.6*	19.1 ± 2.2	30.4 ± 3.7

Values are mean of three replicates ± SD, *p<0.05

and *L. rhamnosus*, a slower growth rate at early time periods as indicated by a prolonged lag and early log phase along with an enhancement in doubling time and a decrease in number of generations were also observed. Later on it followed similar growth pattern as that of respective normal bacteria (Table 1). Almost similar growth patterns with a slight prolongation in lag and early log phase were evident in case of arsenite plus Cr-VI-resistant Lactobacilli strains as compared to their respective normal strains (Fig. 2). All the resistant strains followed entire growth phase patterns very similar to that of their respective normal parent strains when grown either in the absence or in presence of comparatively lower concentrations of corresponding arsenite (up to 4 ppm), Cr-VI (up to 8 ppm) or arsenite plus Cr-VI (up to 4 ppm each) in the media.

Effects on antibiotic sensitivity: To investigate if these metal/metalloid resistant Lactobacilli strains also developed the resistance against antibiotics, the antibiotic sensitivity test of all resistant bacteria was carried out and results for arsenite-resistant bacteria are shown in Fig 3. It was observed that the normal parent strains and the arsenite, Cr-VI and arsenite plus Cr-VI-resistant Lactobacilli strains were sensitive to various tested antibiotics.

Effects on intracellular and membrane enzymes and membrane constituents: To investigate the possible intracellular and membrane alterations following the development of arsenite/Cr-VI-resistance in Lactobacilli strains, various biochemical toxicity parameters were carried out and compared with respective normal parent strains. Intracellular dehydrogenase (DHA) and esterase (EA) activities did not reveal any significant alterations in all the arsenite, Cr-VI and arsenite plus Cr-VI-resistant Lactobacilli strains as compared to their respective normal strains. Results for membrane marker enzymes, namely alkaline phosphatase and Ca^{2+}-Mg^{2+}-ATPase, and membrane structural constituents, namely hexose, sialic acid and phospholipids, are summarized in Table 2, 3. Membrane alkaline phosphatase and Ca^{2+}-Mg^{2+}-ATPase of arsenite, Cr-VI and arsenite plus Cr-VI-resistant Lactobacilli strains did not reveal any major significant change as compared to their respective normal strains. Consequently, the membrane structural constituents also did not show any significant alterations in all arsenite-resistant strains. However, minor significant decline of hexose and sialic acid contents in the range of 20 to 25% were observed in some of the Cr-VI and arsenite plus Cr-VI-resistant strains as compared to their respective normal strains. There was no significant change observed in phospholipid contents in any resistant strain.

***In situ* studies:** In order to evaluate possible synergistic effect of As-III and Cr-VI on membrane enzymes and constituents of major functional intestinal epithelial cells under normal physiologic condition, *in situ* rat intestinal loop incubation exposure individually with arsenite and Cr-VI and arsenite plus Cr-VI in combination were carried out. Over all, a concentration-dependent decline in IEC membrane enzymes and constituents following arsenite, Cr-VI, and arsenite plus Cr-VI *in situ* exposures were observed. Results revealed significant decline of 36 and 26% in alkaline phosphatase activity following 2.0 ppm *in situ* exposures of arsenite and Cr-VI, respectively. Similarly, the decline was 38% in case of arsenite plus Cr-VI (2.0 ppm each). Significant respective decline of 22 and 16% in Ca^{2+}-Mg^{2+}-ATPase activity by arsenite and Cr-VI individually

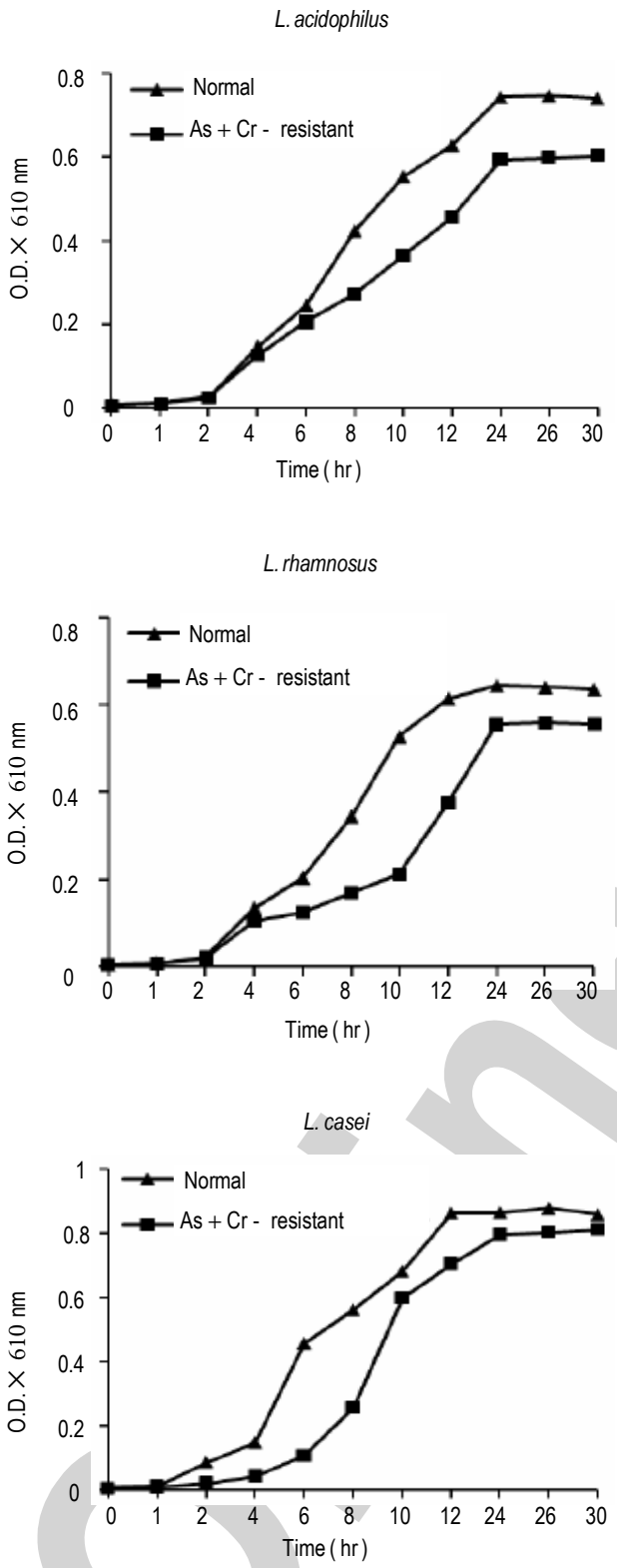

Fig. 2: Growth curves of normal and 32 ppm each arsenite plus Cr-VI-resistant (As + Cr- resistant) *L.acidophilus*, *L.rhamnosus* and *L.casei*. Data represents mean value of five or seven replicates. SD has not been shown to avoid overcrowding. Variance was within a limit of 10-15%

and 26% decline in case of arsenite plus Cr-VI were evident. Likewise, membrane constituents showed significant decline of 47 and 43% in hexose content; and 34 and 12% in sialic acid content following arsenite and Cr-VI individual *in situ* exposures, respectively. Concomitantly, the declines were 51 and 32% respectively, following the exposure of arsenite plus Cr-VI (Fig. 4). There was no significant change in membrane cholesterol and phospholipid contents following arsenite, Cr-VI and arsenite plus Cr-VI *in situ* exposures. In general, effects of arsenite plus Cr-VI in combination were similar to that of arsenite and Cr-VI alone exposures.

The significant finding of the present investigation is the development of arsenite and Cr-VI-resistance in Lactobacilli strains under *in vitro* condition and their similarities in biochemical effects as compared to the respective normal parent strains of *L. acidophilus*, *L. rhamnosus* and *L.casei*. Bacteria are known to develop heavy metal resistance mostly for their survivals, especially the resistance phenomena have been found in the environmental strains. Bacterial plasmids contain specific genes for resistances to various toxic heavy metal ions (Silver and Ji, 1994). Although most of these resistances have been linked to the plasmids, some are chromosomal origins (Silver and Walderhaug, 1992). Developments of resistance against antibiotics in mammals are well known. In order to analyze the ability of newer antibiotics to cause resistance development, studies to develop *in vitro* resistances in bacteria have been documented (Kalenic *et al.,* 1998; Boos *et al.,* 2001; Kim *et al.,* 2003). Uses of probiotic bacteria such as Lactobacilli represent an exciting prophylactic treatment in the prevention of various gastrointestinal disorders including antibiotic-associated diarrhea, infectious bacterial diarrhea, inflammatory and irritable bowel diseases. Probiotics provide protection to intestinal health and are known to convert toxic forms of heavy metal ions into their less toxic forms followed by detoxification (Rolfe, 2000; Isolauri, 2001; Shrivastava *et al.,* 2003). To maintain the survival and functioning of probiotic bacteria under the stress conditions of toxic arsenic and chromium, respective metal/metalloid resistances in Lactobacilli strains have been developed under *in vitro* condition.

The resistance developed in tested strains of Lactobacilli was 32 ppm for arsenite, 64 ppm for Cr-VI, and 32 ppm each in case of arsenite plus Cr-VI. Growth phase studies showed overall similarities in resistant-Lactobacilli strains with that of their respective normal parent strains. Slightly slower growth during lag and early log phase in some of the respective resistant strains as observed when grown in presence of 32 ppm arsenite or Cr-VI, or in 32 ppm each in case of arsenite plus Cr-VI, could be due to the initial adaptation phenomena of respective strains under such higher concentrations. In addition, all the normal parent Lactobacilli strains did not grow when grown in presence of higher than minimal inhibitory concentrations of respective arsenite, Cr-VI or arsenite plus Cr-VI in the media. Furthermore, evaluation of the specificity of arsenite-resistant and Cr-VI-resistant Lactobacilli strains revealed no growth of bacteria when arsenite-resistant strains were grown in presence of higher than minimum inhibitory concentrations of Cr-VI and vice-versa in the medium. Whereas, all arsenite plus Cr-VI-

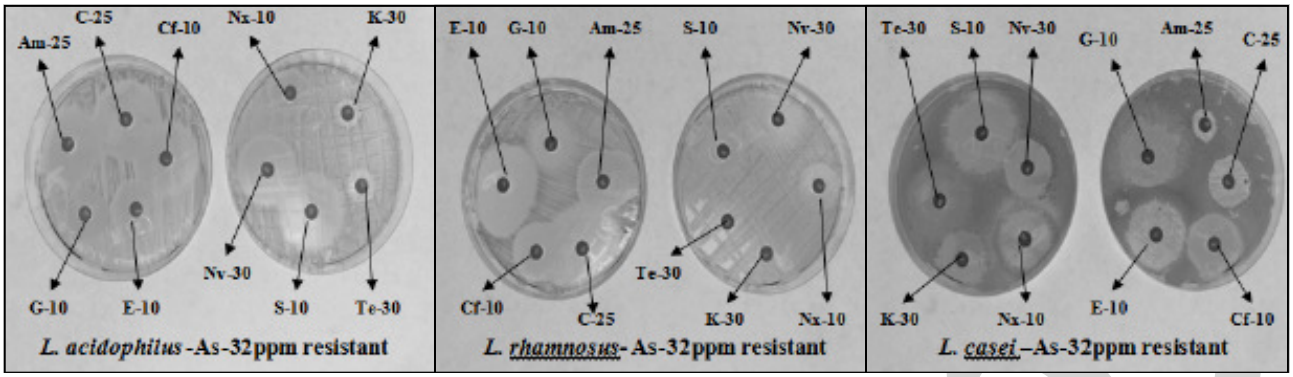

Fig. 3: Antibiotic sensitivity test of arsenite-resistant Lactobacilli strains by disk diffusion method. Am25= Amoxicillin (25 µg); C25= Chloramphenicol (25 µg); Cf10= Ciprofloxacin (10 µg); E10= Erythromycin (10 µg); G10= Gentamycin (10 µg); K30= Kanamycin (30 µg); Nv10= Novobiocin (10 µg); Nx30= Norfloxacin (30 µg); S10= Streptomycin (10 µg); Te30= Teicoplanin (30 µg)

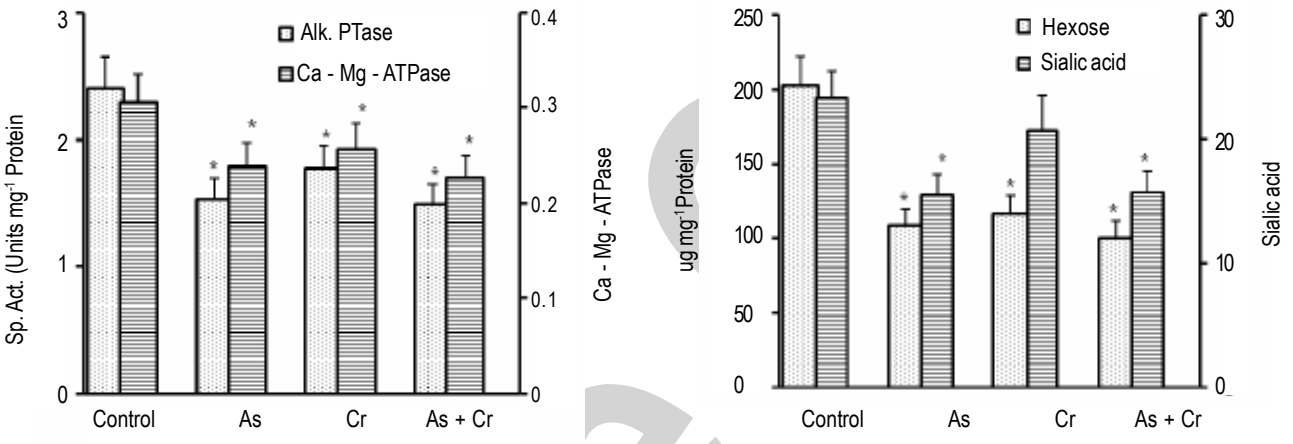

Fig. 4: *In situ* effect of 2 ppm each of arsenite (As), Cr-VI (Cr), and arsenite plus Cr-VI (As + Cr) on rat intestinal epithelial cell [A] Alkaline phosphatase and Ca^{2+}-Mg^{2+}-ATPase and [B] Hexose and sialic acid content. Values are mean of three replicates ± SD. *p<0.05

resistant (32 ppm each) Lactobacilli strains revealed significant growth in presence of either arsenite (32 ppm) or Cr-VI (32 ppm) in the media. The resistance developed tested strains did not grow when grown in presence of other heavy metals such as Pb [$Pb(NO_3)_2$], Hg [$HgCl_2$] or Cd ($CdCl_2$) in the media. Findings indicate that the resistances developed in Lactobacilli strains are specific for arsenite and chromium and their mechanism of resistance development is also different. Further genome- and proteome-wide studies are needed to elucidate the mechanism(s) of development of arsenite and CR-VI-resistance in Lactobacilli.

Heavy metal resistance in a number of different bacteria is known to be present together with antibiotic resistances. There are evidences for possible links between heavy metal and antibiotic resistance in bacteria because these traits are generally associated with transmissible plasmids and the genes are frequently found on the same plasmid. Under environmental conditions of metal stress, such metal and antibiotic resistant population adopts faster by the spread of R-factors than by mutation and natural selection (Matyar *et al.,* 2008; Kamala-Kannan and Lee, 2008). However, in the present study we observed that like their normal parent strains, all the arsenite/Cr-VI-resistant Lactobacilli strains did not acquire resistance against various antibiotics. This indicates that the chronological chronic exposure of arsenite, Cr-VI and arsenite plus Cr-VI during the *in vitro* development of resistance in Lactobacilli did not influence the occurrence of antibiotic resistance.

To elucidate possible biochemical alterations following *in vitro* development of arsenite/Cr-VI-resistance in Lactobacilli strains, various intracellular and membrane associated biochemical parameters were analyzed. The significance of these parameters in the evaluation of toxicity has been documented (Upreti *et al.,* 2007, 2008). Overall findings indicated similarities in all parameters of the resistant Lactobacilli strains as compared to their respective normal parent strains. Minor decline in membrane glucose and sialic acid contents in the arsenite plus Cr-VI-resistant strains in comparison to their respective normal strains may be attributed due to the initial adaptation phenomena in presence of such a high concentrations of arsenite and Cr-VI (32 ppm each) in the media. Overall findings suggest that these resistant Lactobacilli strains may play significant role as probiotics.

Environmental exposure of humans to metals and metalloids is heterogeneous with co-exposure occurring coincident with multiple toxic metal species. The co-exposure of chromium and arsenic can result in a synergistic or depletive response (Nygren *et al.,* 1992). In the present study, *in situ* findings following the exposure of arsenite, Cr-VI, and arsenite plus Cr-VI, under physiologic condition, on rat intestinal epithelial cell membrane enzymes and constituents did not show significant synergistic, additive or depletive response of arsenite plus Cr-VI co-exposure. However, more detailed studies are needed to explore the effects of simultaneous exposures of arsenite and Cr-VI at varying concentrations.

In conclusion, our studies reveal that sequential sub culturing of Lactobacilli strains in arsenite, Cr-VI, or arsenite plus Cr-VI led to the development of respective resistance of these metal/metalloid. The encouraging initial finding is a step towards the novel concept of using arsenite and Cr-VI-resistant Lactobacilli probiotic as a line of defense against arsenic and chromium endemics. However, further studies are needed to understand the mechanisms of resistance, genetic stability, possible transfer frequency of responsible genes to other group of gut residential bacteria, and the conversion of toxic forms of these metal/metalloid ions into less toxic forms followed by detoxification under natural condition.

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