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# A Comparative Study on Rat Intestinal Epithelial Cells and Resident Gut Bacteria (ii) Effect of Arsenite

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**Objective** In order to use facultative gut bacteria as an alternate to animals for the initial gastrointestinal toxicity screening of heavy metals, a comparative study on rat intestinal epithelial cells and resident gut bacteria was undertaken. **Methods** *in vitro* growth rate of four gut bacteria, dehydrogenase (DHA) and esterase (EA) activity test, intestinal epithelial and bacterial cell membrane enzymes and *in situ* effect of arsenite were analysed. **Results** Growth profile of mixed resident population of gut bacteria and pure isolates of *Escherichia coli, Pseudomonas sp., Lactobacillus sp., and Staphylococcus sp.* revealed an arsenite (2-20 ppm) concentration-dependent inhibition. The viability pattern of epithelial cells also showed similar changes. DHA and EA tests revealed significant inhibition (40%-72%) with arsenite exposure of 5 and 10 ppm in isolated gut bacteria and epithelial cells. Decrease in membrane alkaline phosphatase and $Ca^{2+}$-$Mg^{2+}$-ATPase activities was in the range of 33%-55% in four bacteria at the arsenite exposure of 10 ppm, whereas it was 60%-65% in intestinal epithelial villus cells. *in situ* incubation of arsenite using intestinal loops also showed more or less similar changes in membrane enzymes of resident gut bacterial population and epithelial cells. **Conclusion** The results indicate that facultative gut bacteria can be used as suitable *in vitro* model for the preliminary screening of arsenical gastrointestinal cytotoxic effects.

**Key words**: Arsenic; Gut microflora; Intestinal bacteria; Intestinal epithelial cells; Membrane enzymes; GI-toxicity

## INTRODUCTION

Arsenic is a naturally occurring metalloid that exists in inorganic as well as organic forms. The major inorganic forms of arsenic are the trivalent meta arsenite ($iAs^{III}$) and the pentavalent arsenate ($iAs^{V}$). The trivalent arsenic is more toxic than the pentavalent form. Arsenic is the 20th most abundant element in the earth's crust and is widely distributed throughout nature as a result of weathering dissolution, fire, volcanic activity, and anthropogenic input. The majority of humans are chronically exposed to low levels of arsenic, principally through ingestion of food and water and to some extent due to inhalation of arsenic in the ambient air. Drinking water contamination by arsenic remains a major public health problem. The maximum permissible limit of arsenic in water by the WHO is 50 ug/L. High concentrations of arsenic ranging from 200-600 ug/L have been reported in several districts of West Bengal, India[1]. In Bangladesh, about 57 million people have been exposed to arsenic through contaminated wells[2].

Excess intake of arsenic causes irritation of the digestive tract leading to gastrointestinal disorders including pain, nausea, vomiting, and diarrhea. Prolonged ingestion of arsenic can lead to cardiovascular disorders, liver, and kidney injuries, neurological and skin disorders[3]. Chronic ingestion of high levels of inorganic arsenic in drinking water is associated with the increased incidence of human cancer at various sites such as skin, lung, bladder and other internal organs[4-5]. However, in animals its carcinogenic effects are not well established.

The gastrointestinal (GI) tract presents itself as the first organ susceptible to attack by ingested xenobiotics consequently, concentrations that must be endured by this tissue are often many times higher than those endured by other tissues[6]. Though oral uptake is one of the major routes of exposure to arsenic for the general population, comprehensive data concerning intestinal toxicity of inorganic arsenic are lacking. Bacteria living within the intestinal lumen are known to play an important role in host homoeostasis. The constant interaction between the host and its microbial guests can infer

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important health benefits[7]. Thus, the metabolism of inorganic arsenic by the gastrointestinal flora may have considerable significance in humans and animals. Available literature is scanty regarding the interaction of inorganic arsenic with gut microflora as well as rat intestinal epithelial cells and needs further exploration. Recently, much interest has been focused on developing *in vitro* toxicity tests evaluating their usefulness in predicting toxicities. Studies on microorganisms isolated from rat gut would be significantly important. The present study was undertaken to compare the effects of arsenic toxicity on the intestinal epithelial cells with those on the resident facultative intestinal bacteria.

## MATERIALS AND METHODS

### *Animals and Chemicals*

Sodium meta-arsenite (trivalent arsenic; As-III) purchased from Sigma-Aldrich Comp. USA, was of analytical grade. Arsenic solution containing various concentrations of As (III) was prepared in water. Other chemicals purchased from Sigma-Aldrich, E. Merck, Qualigens, India and Hi-Media, India, were of analytical grade. Male Wistar rats weighing 200±20 g were procured from ITRC Animal Breeding Facility, and maintained on standard pellet diet and water *ad libitum*. Prior to sacrifice by cervical dislocation, the animals were fasted overnight with free access to drinking water and the following were investigated.

### *Isolation of Rat Intestinal Bacteria*

The cecum was located by opening the abdomen with full aseptic precautions and injected with 5.0 mL of sterilized phosphate buffered saline (PBS) from one end. After 2 min fluid from the cecum was collected and one loop-full was streaked out on a nutrient agar plate. After incubation for 24 h the bacterial colonies formed were studied and the smears were stained with Gram stain. The biochemical reactions were put up and the bacteria were identified on the basis of the Bergey's Manual of Determinative Bacteriology[8]. The bacteria selected for further study were *E. coli*, *Pseudomonas sp*., *Lactobacillus sp*., and *Staphylococcus sp*. To isolate the mixed population of resident bacteria the cecum contents were filtered twice through sterile glass wool to remove the intestinal debris and the fecal matter. The mixed bacterial cells were harvested by centrifugation at 10 000 g for 20 min at 4℃, washed twice with desired media and resuspended in the same media/buffer. The log phase cultures of bacteria were inoculated in nutrient broth containing 2.0 (g/lit) peptone, 3.0 beef extract, 5.0 NaCl, and incubated at 37℃ on a rotatory shaker.

### *Isolation of Rat Intestinal Epithelial Cells*

Intestinal epithelial cells were prepared by the method of Weiser[9]. In brief, the small intestines were flushed gently with normal saline containing 1.0 mmol/L dithiothreitol. The cecal end of the intestine was ligated and solution 'A' containing 1.5 mmol/L KCl, 96 mmol/L NaCl, 27 mmol/L sodium citrate, 8 mmol/L $KH_2PO_4$, 5.6 mmol/L $Na_2HPO_4$ (pH 7.3) was filled after the other end was clamped with artery forceps. The intestine was immersed in solution 'A' and incubated at 37℃ for 15 min in a constant temperature shaker bath. The intestine was emptied, fluid was discarded and filled with solution 'B' containing 1.5 mmol/L EDTA and 0.5 mmol/L dithiothreitol in PBS (pH 7.2) and immersed in solution 'A' for incubation. After incubation, the contents were emptied into a plastic centrifuge tube to recover the first epithelial cell population. The process of filling with solution 'B' and collecting the washing was repeated at different time points of incubation (2, 2, 3, 4, 5, 7, and 10 min, respectively). Fractions 1 to 4 were pooled and designated as 'Upper Villus'.

### *Effects of Arsenic on Growth of Bacteria*

Arsenite salt solution was added to the final concentrations of 2 ppm to 20 ppm in the media. Growth was measured at different time intervals up to 30 h by turbidimetry at 610 nm. Growth measurements were carried out and the specific growth rates of the bacteria were calculated as described by Espigares and Mariscan[10]. For viability testing serial 10-fold dilutions of sample were prepared in sterile normal saline. Duplicate 100 µL samples were plated on nutrient agar plates and incubated at 37℃ for 24 h and then the colonies were counted.

### *Effects of Arsenic on Viability of Intestinal Epithelial Cells*

A single cell suspension of the intestinal epithelial cells ($2\times10^6$ cells/mL) was set up in triplicate with or without arsenic ranging from 2 ppm to 20 ppm in 96-well plates and incubated for 24 h at 37℃. After incubation 20 µL of MTT (5 mg/mL PBS) was added in each well. Following incubation for 4h at 37 ℃ the medium was aspirated and 200 µL of DMSO was added to all wells and mixed thoroughly. After a few minutes the plates were read on a Micro-Elisa reader at the

wavelength of 570 nm[11].

*Effect of Arsenic on Dehydrogenase Activity (DHA) of Intestinal Bacteria and Epithelial Cells*

Dehydrogenase activity was tested by the method as described by Liu[12]. With minor modifications in brief, cell suspensions in 0.025 mol/L isotonic phosphate buffer containing $1.6\times10^6$ cells/mL were prepared. Two milliliters of cell suspension was incubated with 1.0 mL resazurin (50 mg/liter in phosphate buffer), arsenite solution was added to the final concentration of 0-20 ppm and sterile deionized water was added to a final volume of 6.0 mL. Tubes without arsenite were used as control. All the tubes were incubated for 5 h at 20℃. The test was stopped after 5 h by adding 0.25 mL 1.0% $HgCl_2$ solution, followed by centrifugation at 2500 g for 25 min. DHA activity was determined spectrophotometrically in the supernatant at 601 nm before and after incubation.

*Effect of Arsenic on Esterase Activity (EA) of Intestinal Bacteria and Epithelial Cells*

Esterase activity test was determined following the slightly modified method described by Obst and Holzapfel-Pschorn[13]. Three milliliters of cell suspension ($1.6\times10^6$ cells/mL) was incubated with 0.01 mL fluorescin diacetate (FDA) solution (10 mg FDA/mL acetone) without or with arsenite and sterile deionized water to a final volume of 6.0 mL at 20℃ for 5 h under constant agitation. Reaction was stopped after 5 h by adding 3.0 mL acetone, followed by centrifugation at 2500 g for 25 min. Esterase activity in supernatant was determined by the formation of free fluorescin, which was measured spectrophotometrically at 490 nm. A mixture of water and acetone (1:1) was used as a photometric blank.

*Studies on Intestinal Epithelial and Bacterial Cell Membrane*

*in vitro Studies* An approximately equal number of bacterial and intestinal epithelial cells ($2\times10^6$ cells/mL) were exposed to different final concentrations of arsenite (0-10 ppm) in the media at 37℃ for 24 h with constant shaking. Cells were harvested by centrifugation (10 000 g, 15 min, 4℃) and cell membrane was prepared as described by Kumar and Upreti[14].

*in situ Studies* Laparotomy on each rat was performed by midline incision under light ether anesthesia. The intestine was exteriorized, washed with normal saline, using a syringe and a blunt needle, through two small cuts. One cut was made slightly distal to the duodeno-jejunal junction and another at the distal end of the ileum. After washing, the opening was ligated and a 30-cm-length loop was prepared from the upper end of the intestine using sterile threads. Arsenite solution was administered into the loop through a proximal opening, which was then immediately ligated. Control loops contained normal physiologic saline solution[15]. Arsenite solution was also administered into another loop prepared between 2.0 cm above and 2.0 cm distal to the cecum. Control cecal loops contained physiologic saline solution. The whole intestine was kept *in situ* and the abdomen was stitched immediately. Proper breathing and anesthesia of the animal were maintained throughout the experiment. Intestinal and cecal loops were removed after 30 min incubation, epithelial cell brush border membrane and bacterial cell membrane were prepared.

*Preparation of Bacterial Cell Membrane*

The bacterial cells were harvested by centrifugation (10 000 g, 15 min, 4℃) in the early stationary phase of growth, washed twice with 30 mmol/L Tris buffer (2.5 mmol/L EDTA, pH 8.1) and resuspended in the same buffer. Lysozyme was added to the final concentration of 200 ug/mL to prepare spheroplasts and cells were incubated for 30 min at 25℃. All subsequent steps were carried out at 0℃-4℃. Spheroplasts were collected by centrifugation at 15 000 g for 15 min, resuspended in Tris buffer and disrupted by four 15-sec bursts with ultrasonic processor and centrifuged at 1000 g for 10 min to remove debris and unbroken cells. The resulting supernatant consisting of membrane and cytoplasmic fractions was centrifuged at 50 000 g for 60 min. The pellet consisting of both outer and inner membrane envelopes was washed twice and resuspended in buffer for enzyme assays and biochemical analysis[14].

*Preparation of Rat Intestinal Epithelial Cell Membrane*

The brush border membrane (BBM) was prepared by the method of Forstner *et al.*[16]. The small intestine was removed and gently washed thrice with ice-cold normal saline to remove the food materials. The intestine was inverted and the epithelial layer was scraped off with the help of a glass slide. The cell scraping was weighed, placed in 75 volumes of 5.0 mmol/L EDTA, adjusted to pH 7.4 with sodium hydroxide (EDTA buffer), and homogenized in ultra-sonicator. After the homogenate was centrifuged at 450 g for 10 min at 4℃, the sediment was washed thrice with 5 volumes of EDTA

buffer. The washed sediment of the crude brush border fraction was then suspended in 2 volumes of 90 mmol/L sodium chloride and 0.8 mmol/L EDTA buffer, mixed thoroughly and kept (20 min to 30 min) until a well defined sediment developed. The supernatant and sediment were poured in succession through a pad of glass wool to remove aggregated particles. After the glass-wool pad was washed with a further 20 mL of 5 mmol/L EDTA buffer, the brush borders from the total washing were sedimented by centrifugation at 450 g for 10 min and washed once with 2.5 volumes of 2.5 mmol/L EDTA buffer. The final pellet was suspended in a volume of 2.5 mmol/L EDTA buffer and used for estimation of biochemical parameters.

*Enzyme Assays and Biochemical Estimations in Bacterial and Epithelial Cell Membranes*

Alkaline phosphatase was determined according to Weiser[9] and $Ca^{2+}$-$Mg^{2+}$-ATPase as described by Hidalgo *et al.*[17]. Enzyme units were defined as micromoles of the products formed or liberated per minute under the assay conditions. Specific activity was expressed as units per milligram protein. Protein was determined according to Lowry *et al.*[18] using bovine serum albumin as standard.

*Statistical Analysis*

The results were expressed as $\bar{x} \pm s$. Comparisons were made with appropriate controls employing Student's *t*-test. $P < 0.05$ was considered statistically significant.

## RESULTS

*Effects of Arsenite on Growth of Rat Gut Bacteria*

The *in vitro* toxicity of arsenite on the growth pattern of mixed resident rat gut bacteria isolated from cecum and pure isolates of *E. coli, Pseudomonas sp., Lactobacillus sp., and Staphylococcus sp*. is shown in Fig. 1. Growth profile of gut bacteria in general revealed an arsenite concentration-dependent inhibition. Isolates of *E. coli* and *Pseudomonas sp*. belonging to Gram-negative group when grown in media with or without arsenite showed a more or less similar growth profile as that of mixed resident gut bacteria. In comparison to respective controls, the arsenite exposed Gram-negative bacteria along with mixed bacteria exhibited a prolonged lag phase before becoming accommodated to arsenite stress. The presence of 20 ppm of arsenite in the growth media was detrimental to the growth. More or less similar growth profiles were observed with Gram-positive bacteria when grown in the presence of arsenite. However, the two Gram-positive bacteria studied did not reveal a prolonged lag phase and were well accommodated to arsenite stress. *Lactobacillus sp*. could grow well even in the presence of 20 ppm arsenite concentration.

*Effects of Arsenite on Cell Viability*

Cell viability of the mixed resident gut bacteria following 24 h *in vitro* exposure to arsenite revealed a concentration-dependent reduction. Concomitantly, the viability pattern of intestinal epithelial cells following MTT assay also revealed a similar concentration-dependent cell death percentage. Arsenite concentration of 10ppm or more caused 60%-95% cell death in both types of the cells (Fig. 2).

*Effects of Arsenite on Dehydrogenase Activity (DHA) and Esterase Activity (EA) Tests*

DHA and EA tests revealed a significant arsenite concentration-dependent inhibition in both types of the cells as compared to their respective controls. In case of isolated rat epithelial cells, a decrease of 40%-72% was observed with arsenite exposure of 5 ppm and 10 ppm, respectively. A more or less similar pattern of decrease in DHA was observed in the isolated gut bacteria following arsenite exposure. Decrease in DHA of isolated gut bacteria was comparatively less in Gram-positive bacteria. Inhibition pattern of EA in both types of the cells following arsenite exposure was also similar to that of DHA (Fig. 3).

*in vitro Effects of Arsenite on Membrane Enzymes*

Both Gram-positive and Gram-negative bacterial cells grown in media without or with arsenite for 24 h, revealed a concentration-dependent decrease in their membrane enzyme alkaline phosphatase and $Ca^{2+}$-$Mg^{2+}$-ATPase activities as compared to their respective controls. Decrease in activity of the two enzymes was 33%-55% in all the four isolated resident gut bacteria with 10 ppm arsenite exposure. The isolated intestinal epithelial villus cells when exposed to different arsenite concentrations in media for 24 h also revealed a concentration-dependent decrease in their membrane enzyme activities. A decline of 17% and 19% was observed in alkaline phosphatase and $Ca^{2+}$-$Mg^{2+}$-ATPase activity with 2 ppm arsenite exposure, whereas it was 65% and 60% respectively with 10 ppm arsenite exposure (Fig. 4).

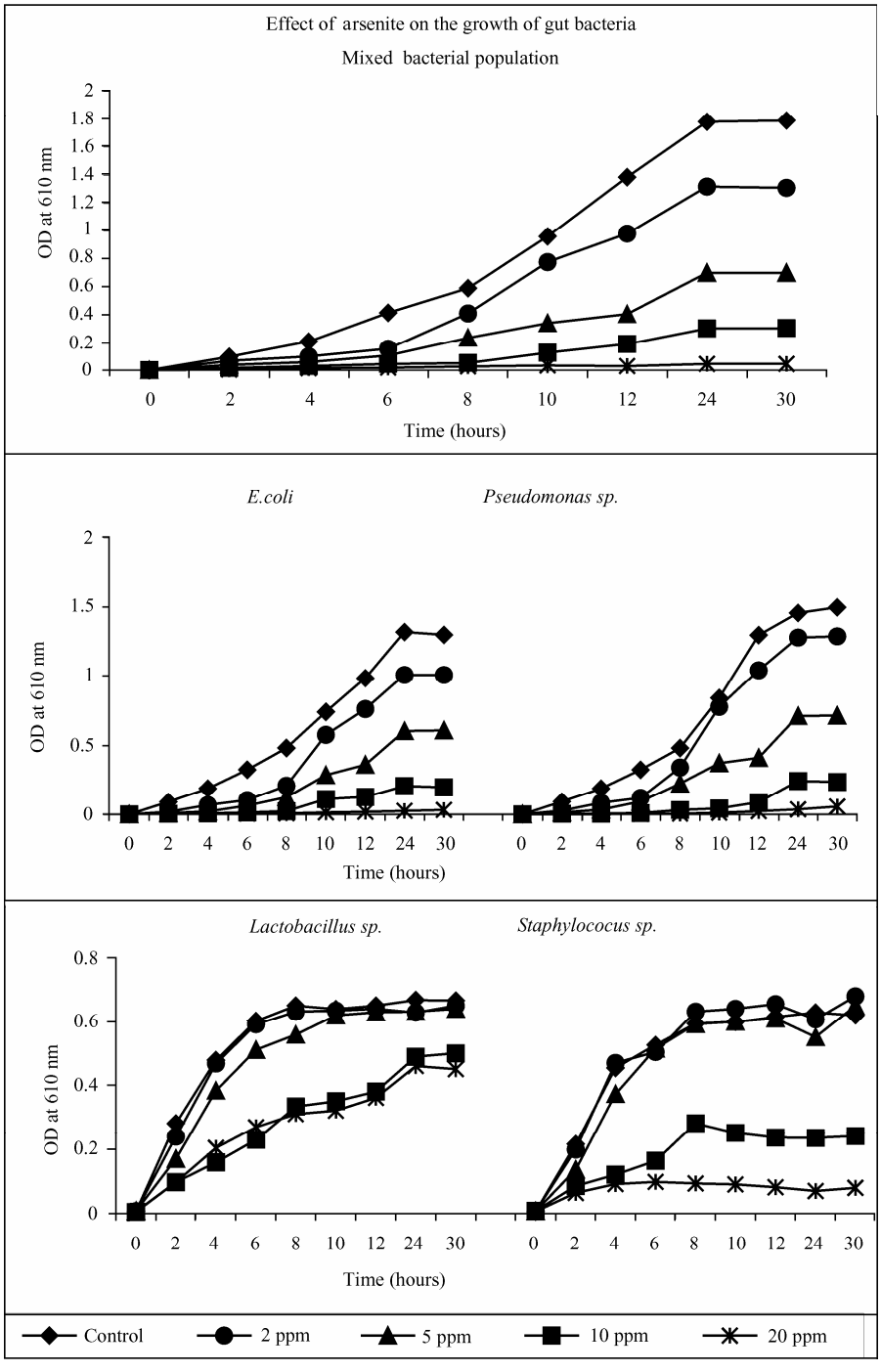


FIG. 1. Growth profile of mixed bacterial population and four facultative bacteria isolated from normal rat cecum and grown in media without or with arsenite. The data represent the mean value from 3-5 rats. S.D. has not been shown to avoid overcrowding. Variance was within a limit of 10%-15%.

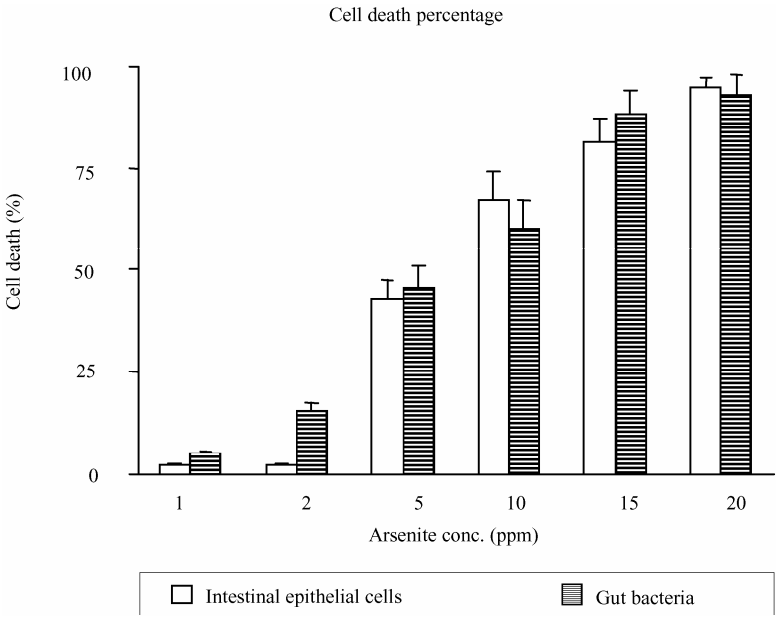


FIG. 2. Cell viability of mixed resident gut bacteria and epithelial cells following 24 h *in vitro* exposure to arsenite.

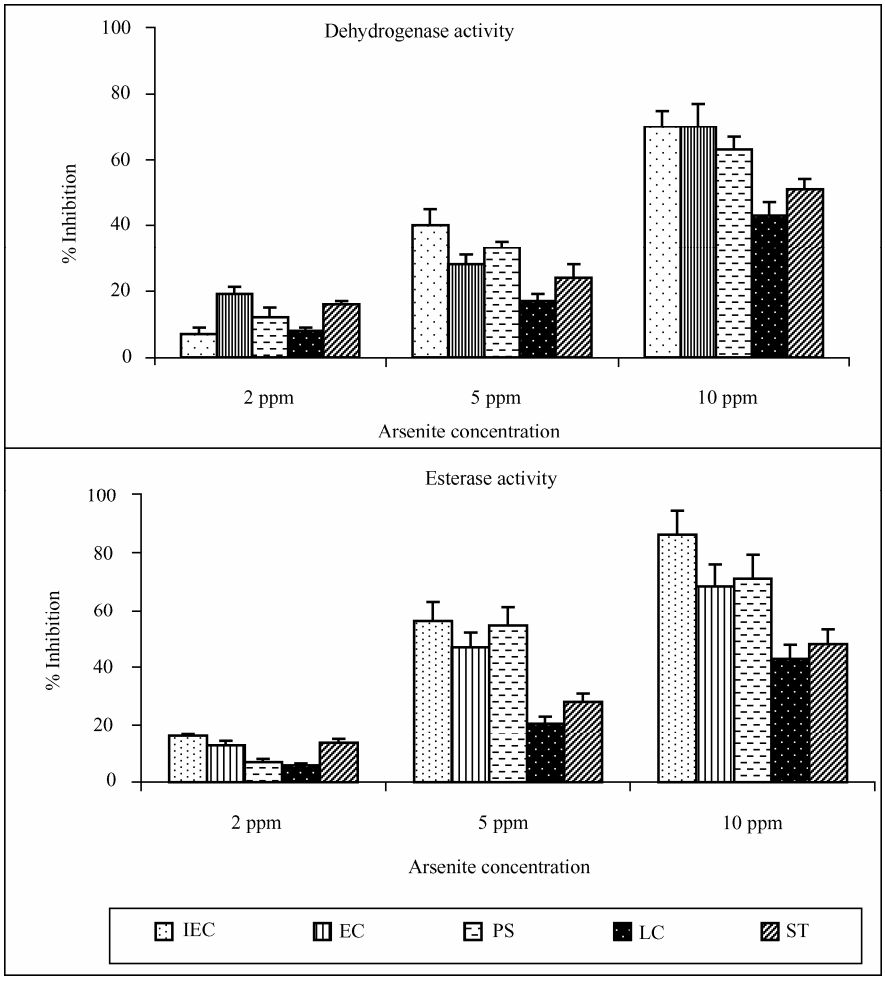


FIG. 3. Inhibition of dehydrogenase and esterase activity of intestinal epithelial cells (IEC) and intestinal bacteria (EC-*E. coli*; PS-*Pseudomonas sp.*; LC-*Lactobacillus sp.*; ST-*Staphylococcus sp.*) following *in vitro* exposure of arsenite. Values are $\bar{x} \pm s$ from three set of experiments.

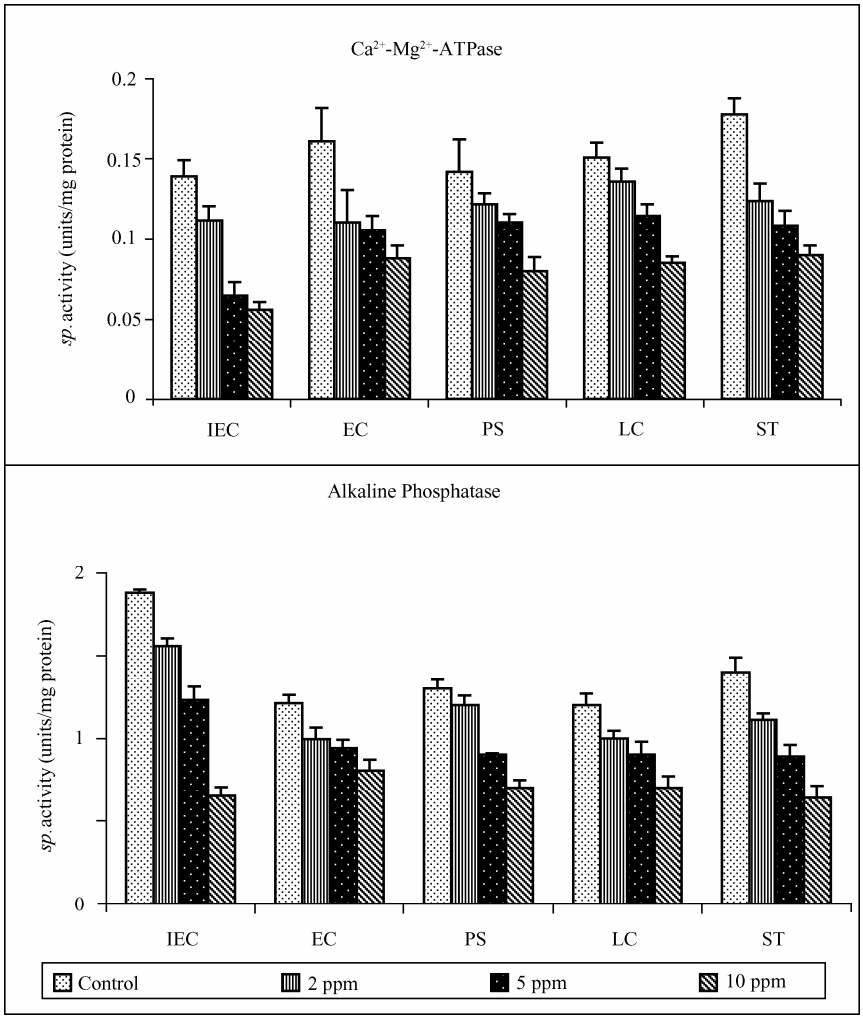


FIG. 4. *In vitro* effect of arsenite on membrane enzymes of intestinal bacteria and epithelial cells. Cells were exposed to various concentrations of arsenite in the media at 37°C for 24 h.

### *in situ Effects of Arsenite*

Thirty-minute *in situ* incubation of 2, 5, and 10 ppm arsenite concentrations using intestinal loop model revealed a significant decrease of 37%, 52%, and 67% respectively, in alkaline phosphatase activity of rat intestinal epithelial cell brush border membrane. As compared to the control the decline in $Ca^{2+}$-$Mg^{2+}$-ATPase activity was 21%, 31%, and 41%, respectively. Concomitantly, with 5 ppm and 10 ppm arsenite *in situ* incubated resident bacteria also showed a significant decline of 14% and 43% in alkaline phosphatase and 38% and 41% in $Ca^{2+}$-$Mg^{2+}$-ATPase activities respectively in their membrane preparations. However, with 2 ppm arsenite incubation the decline in activity of the two enzyme activities in membrane preparations of mixed bacteria was not significant (Fig. 5).

## DISCUSSION

The significant finding of the present study is the striking similarity of toxic effects of arsenic on the resident intestinal bacteria and the intestinal epithelial cells of rats. A single cell layer thick intestinal epithelium organized in a villus architecture, spans a large surface area and functions in intimate contact with prokaryotes existing at densities of up to $10^{11}$ organisms/mL of luminal contents[19]. The mammalian host provides a thermostable and nutrient-rich environment for intestinal bacteria while exploiting their presence to derive a variety of benefits. For example, the human enteric flora provides for the metabolism of bile acids, bilirubin, cholesterol and short-chain fatty acids and assists in the synthesis of nutrients such as vitamin K. In general, gut bacteria have important and specific metabolic, trophic, and

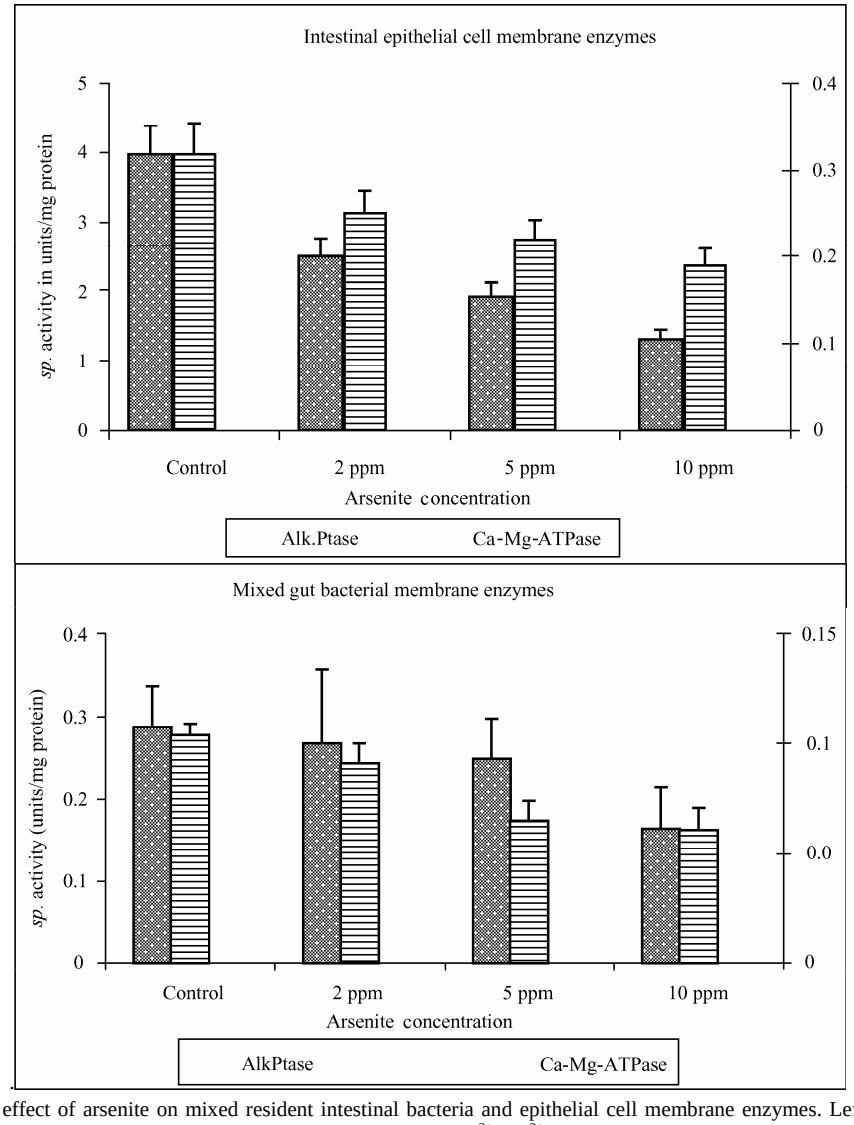


FIG. 5. *in situ* effect of arsenite on mixed resident intestinal bacteria and epithelial cell membrane enzymes. Left axis denotes specific activity for alkaline phosphatase and right axis for $Ca^{2+}$-$Mg^{2+}$-ATPase. Values are $\bar{x} \pm s$ from three rats. Other details are given in the text.

protective functions[20]. Molecular analysis of commensal host-bacteria relationship in the intestine has shown that the differentiation of epithelial cells is greatly affected by interaction with resident bacteria[21]. The resident gut bacteria and intestinal epithelial cells are known to interact in harmony[22-23].

Growth of bacteria is a common parameter to study the response to toxic insults, as it reflects directly the viability of the bacterial population, while several methods are available to study the viability of eukaryotic cells. In the present study, the growth profile of gut bacteria in general revealed an arsenite concentration-dependent inhibition. The arsenite exposed Gram-negative bacteria exhibited a prolonged lag phase while in the Gram-positive bacteria studied, the lag phase was similar to that of controls. Concomitantly, the viability pattern of the intestinal epithelial cells also revealed a similar arsenite concentration-dependent cell death.

DHA is linked to bacterial respiration and EA is involved in intra- and extra-cellular degradation of organic matters. Both parameters have been used to study the toxicity of various chemicals in bacteria[24]. A significant concentration-dependent inhibition of DHA and EA was observed in the intestinal epithelial cells and the bacteria following arsenite exposure. Decrease in the activity of the enzymes was less in Gram-positive bacteria.

Cell membrane is the first site of interaction following the exposure to a chemical. The uptake and

exchange of different ions take place through the intestinal mucosa. Inhibition of membrane transport enzymes like alkaline phosphatase and $Ca^{2+}$-$Mg^{2+}$-ATPase may influence the transport of $PO_4^-$, $Ca^{2+}$, $Mg^{2+}$, and other vital ions in the intestine. In the present study, these membrane marker enzymes were also analyzed and compared between the intestinal epithelial and bacterial cells following *in vitro* arsenite exposure. A more or less similar arsenite concentration-dependent decrease in the membrane transport enzymes of intestinal facultative Gram-positive and Gram-negative bacteria and intestinal epithelial cells was evident. In addition, the *in situ* effect of arsenite on membrane enzymes of the mixed population of intestinal bacteria and epithelial cells validated the *in vitro* findings, thus further strengthening the dynamic relationship between eukaryotic and prokaryotic cells in the gastrointestinal tract.

It is also known that all living organisms detoxify arsenic by various mechanisms, such as uptake of arsenate by phosphate transporters and aquaglyceroporins, conversion of arsenate to arsenite by arsenate reductases, and extrusion or sequestration of arsenite. Bacterial plasmids encode resistance systems for toxic metal ions, including $AsO_2^-$, $AsO_4^{(3-)}$. The function of most resistance systems is based on the energy-dependent efflux of toxic ions. Some of the efflux systems are ATPases and others are chemiosmotic cation/proton antiporters. The arsenic resistance efflux system transports arsenite, alternatively using either a double-polypeptide (ArsA and ArsB) ATPase or a single-polypeptide (ArsB) functioning as a chemiosmotic transporter. The third gene in the arsenic resistance system, ArsC, encodes an enzyme that converts intracellular arsenate to arsenite, the substrate of the efflux system. Besides the widely spread plasmid arsenic resistance determinants, some bacteria possess the ability to enzymatically oxidize arsenite to less toxic arsenate[25-26]. In mammalian cells acquired arsenic resistance is associated with overexpression of P-glycoprotein and can be reversed by PSC833, an inhibitor for P-glycoprotein. The P-glycoproteins are involved at least in part, in arsenic efflux in mammalians[27]. Therefore, living organisms may adapt to toxicity of arsenicals. Biochemical mechanisms of resistance may vary between prokaryotic and eukaryotic cells, but active efflux of arsenicals is one of the most common phenomena[28-30]. While the overall schemes for arsenic resistance are similar in prokaryotes and eukaryotes, some of the specific proteins are the products of separate evolutionary pathways[31]. Therefore, in the present study it is not surprising to find similar responses by the two diverse types of cells to toxic insult by arsenic. Furthermore, bacteria capable of either oxidizing As (III) or reducing As (V) coexist and are ubiquitous in soil environments, suggesting that the relative abundance and metabolic activity of specific microbial populations play an important role in the specialization of inorganic As in soil pore water[32]. This could be true with the bacterial population of intestines and remains to be investigated, as there are only few reports on the interaction of intestinal bacteria and arsenicals. On the basis of indirect evidence, Rowland and Davies[33] suggested that in the presence of caecal contents, small amounts of methyl arsonic acid and dimethylarsinic acid are formed. Hall *et al.*[34] reported that mouse intestinal caecal microflora are a high capacity methylation system that might contribute significantly to methylation of inorganic arsenicals in intact animals. Further studies to understand the role and specificity of resident gut bacteria in the detoxification of heavy metals by gastrointestinal tract would shed more light in revealing the inter-relationship between intestinal bacteria and intestinal epithelial cells.

Bacteria are suitable for toxicity testing, as they are easy to handle and respond quickly in measurable way. Furthermore, most biochemical pathways present in bacteria are similar to those in higher animals and thus, sharing the mechanisms of response to toxic substances as observed in the present study. This besides supporting the findings of our earlier study[23], further strengthens our belief that facultative gut bacteria can be used as an alternate to animals, at least for the preliminary screening of heavy metal toxicity in gastrointestinal tract.

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